PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: HUMAN NEUROPEPTIDE Y/PEPTIDE YY RECEPTOR OF THE Y1-TYPE AND ANTISENSE OLIGONUCLEOTIDES THERETO WHICH INHIBIT VASOCONSTRICTION

- ● PYY 1-36
- ○ NPY 1-36
- □ [Leu^{31}, Pro^{34}]NPY
- ■ NPY 2-36
- ▽ C2-NPY
- ▼ PP
- ▲ NPY 13-36
- △ NPY 18-36

y-axis: ^{125}I-PYY Bound (% of B_0)

x-axis: log[competitor] (M)
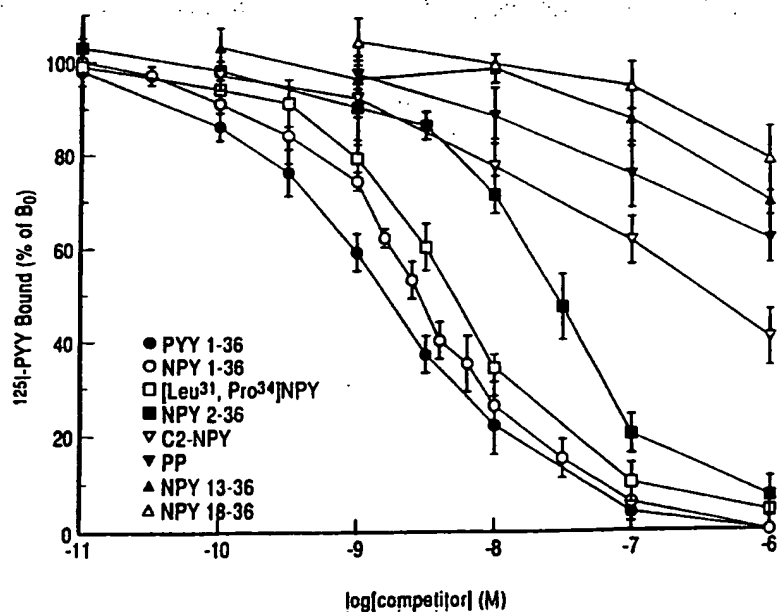
(57) Abstract

The present invention is directed to the cloning, identification and uses of the human Y-1 type neuropeptide Y/peptide YY receptor. The isolated DNA clone is expressed in COS1 cells for ligand binding competition assay. Also described is a new principle for the development of an inhibitor of the contractile responses of neuropeptide Y in human blood vessels by the use of an antisense oligodeoxynucleotide complementary to the human Y-Y1 receptor mRNA.

1

HUMAN NEUROPEPTIDE Y/PEPTIDE YY RECEPTOR
OF THE Y1-TYPE AND ANTISENSE OLIGONUCLEOTIDES THERETO
WHICH INHIBIT VASOCONSTRICTION

Under United States patent practise, this application for Letters
Patent is a Continuation-in-Part application of my earlier United
States Patent Application 891,453, filed May 29th 1992.

Neuropeptide Y (NPY) and peptide YY (PYY) are structurally related
peptides that primarily function as neurotransmitter and
gastrointestinal hormone, respectively. Previous functional and binding
data have indicated the existence of at least three distinct receptor
types, Y1, Y2, and Y3, for NPY and/or PYY in mammals. We describe here
a human Y1 cDNA clone, hY1-5, isolated from a fetal brain library. The
human Y-1 receptor consists of 384 amino acids and has seven putative
transmembrane (TM) domains like other members of the G-protein-
coupled superfamily of receptors. In the region spanning the TM
domains, the Y-1 receptor displays 29% sequence identity to human
tachykinin receptors, but it only shows 21% and 23% homology with
proposed bovine (LCR1) and Drosophila (PR4) NPY-receptor clones,
respectively. Northern blot analysis of a human neuroblastoma cell
line, SK-N-MC, previously used by many investigators as a model
system for studies on the Y-1 receptor, revealed a single 3.5 kb mRNA
species. Reverse transcriptase analysis (RT-PCR) indicated expression
also in human cultured vascular smooth muscle cells, supporting the
view that the Y1-receptor is associated with NPY/PYY-evoked
vasoconstriction. When expressed in COS1 cells, hY1-5 conferred
specific ^{125}I-PYY binding sites with displacement patterns
characteristic of the Y1-receptor, i.e. PYY ≥ NPY ≥ [Leu^{31}, Pro^{34}]NPY >>
NPY2-36 > C2NPY > pancreatic polypeptide > NPY13-36 > NPY18-36.
Moreover, in the Y1-receptor transfected COS1 cells, but not in type 1
angiotensin II receptor transfected control cells, NPY and PYY

accelerated $^{45}Ca^{2+}$ influx and inhibited forskolin-stimulated cAMP accumulation, both phenomena being characteristic of the mammalian Y-1 receptor.

Neuropeptide Y (NPY) is a tyrosine-rich 36-amino acid peptide
5   with a carboxyterminal amide which displays a remarkable degree of structural conservation in evolution. It is one of the most abundant and widely distributed neuropeptides within the central nervous system and belongs to a peptide family that also includes peptide YY (PYY) and pancreatic polypeptide (PP). Mammalian NPY and PYY show 70%
10   sequence identity while PP is 50% homologous to NPY and PYY. NPY is widely distributed in the brain, notably in "limbic" regions, and the peripheral nervous system, and is often co-localized with norepinephrine, e.g. in sympathetic perivascular nerve fibers,supplying the cardiovascular system [see Trends in Pharmac. Sci. 8:231 (1987)].
15   In the brain many effects, including stimulation of appetite, anixiolysis/sedation and modulation of pituitary hormone release, have been attributed to NPY/PYY. Among the many peripheral actions of NPY, it has been suggested to be involved in a large number of neuroendocrine functions, stress responses, circadian rhythms, central autonomic
20   functions, eating and drinking behavior, and sexual and motor behavior; most attention has been given to its vasoconstrictor effects. In addition, it is also possible that NPY is related to various neurological and psychiatric illnesses such as Huntington's Chorea, Alzheimer's disease, and major depressive illness. However, in the absence of
25   specific receptor antagonists, functional studies and receptor characterizations have been difficult to perform.

Based on functional and binding data obtained from studies of various organs and cell types, it has previously been suggested that NPY/PYY receptors are heterogeneous [see Ann. NY Acad. Sci. 611:7
30   (1990); Regul. Pept.12:317 (1986); and Life Sci. 50: PL7 (1992)] and the nomenclature "Y1-, Y2- and Y3-receptor type "was introduced to encompass this heterologous nature. The Y1-receptor binds NPY and PYY with similar affinity, as well as the synthetic analog [Pro^{34}]NPY and analogs thereof, but C-terminal fragments of NPY and PYY have been

shown to bind poorly. In contrast, while the Y2-receptor also binds NPY and PYY with high affinity, the C-terminal fragments, e.g. NPY13-36,as well as a centrally truncated analog C2NPY [see Ann. NY Acad Sci. 611:35 (1990)], are only slightly less potent than the intact peptides at

5   this receptor type. More recently, data from several laboratories [see Trends Pharmacol. Sci. 12:389 (1991)] have indicated the existence of a Y3-type of receptor, whose main characteristic is that PYY shows markedly lower affinity than NPY.

     In order to address the structural and functional relationships of

10   the NPY/PYY receptors the present invention pursued the isolation of receptor DNA clones using several strategies. These strategies led to the cloning of a putative human Y1-receptor cDNA clone. This clone, hY1-5, appears to be a human homolog of a previously published rat "orphan" receptor, FC5 [see FEBS Lett. 271:81 (1990)]. The latter rat

15   clone had appeared relevant to the present invention because its expression pattern, as studied by *in situ* hybridization,was reminiscent of that of the Y1-receptor protein, as shown by receptor autoradiography. Thus, a polymerase chain reaction (PCR) product was generated corresponding to the rat "orphan" receptor. Using this,

20   homologous human cDNA clones were isolated.

     It is, accordingly, one aspect of the present disclosure is to present functional evidence identifying one such clone as a human NPY/PYY receptor of the Y1-type.

     With the successful cloning of the NPY-Y1 receptor as described

25   herein for the first time, and on the basis of the predicted mRNA sequence, another aspect of the present invention is to describe an 18-base antisense oligodeoxynucleotide sequence that corresponds to a coding region near the human Y1-receptor amino-terminus.

     Still a further aspect of the present disclosure is to describe the

30   inhibitory effect of treatment with neuropeptide Y-Y1 receptor antisense oligodeoxynucleotide on the contractile response to NPY of human arteries and veins.

     The following figures, disclosure and examples are provided to allow one to receive a more complete understanding of the present

4

invention. These examples are not intended nor provided to limit the scope of the present invention in any manner, and it would be improper for one to interpret them as doing so (for example although Example VI depicts only a single antisense sequence, hY1-AS, this sequence is to be
5   considered as merely a specific example of a class of antisense sequences which have similar capabilities of affecting the NPY-evoked contractile response of blood vessels as described herein).

With reference to the accompanying figures,

Figure 1 is a side-by-side comparison of Northern and Southern
10 hybridizations;

Figure 2 depicts ligand competition for ^{125}I-PYY binding in hY1-5 (Y1-receptor) transfected COS1 cell membranes;

Figure 3 depicts the contractile effect of NPY on human subcutaneous arteries; and
15   Figure 4 depicts the contractiel effect of NPY on human veins.

More specifically, as will be described in detail below, Figure 1 depicts the Northern blot of human neuroblastoma cell lines probed with a human Y1 fragment, each lane containing 15 μg of total RNA. Figure 1 also depicts the Southern blot of human genomic DNA under
20 conditions of high stringency, with each lane containing 10 μg of genomic DNA.

With regard to Figure 2, the competition data are presented as a percentage of specific binding in the absence of competitor wherein each point is the mean ± SEM of two triplicate experiments. The
25 concentration of ^{125}I-PYY was 0.1nM. Each tube contained membranes (crude particulate fractions) from 2X10^6 COS1 cells. Non-specific binding was defined as binding in the presence of 1μM unlabeled NPY.

With respect to Figures 3 and 4, as described above, these figures depict the contractile effect of NPY on human subcutaneous (Figure 3)
30 arteries (diameter of 0.41 ± 0.03 mm) and (Figure 4) veins (diameter of 0.43 ± 0.03 mm) expressed as a percent of the contraction induced by 60 mM KCl. To obtain the data depicted, all vessels were incubated at 37° C for 48 hours. The symbols indicate treatment with (•) 1 μM antisense, (O) 1 μM sense, (I) 1 μM mismatch or (/) control, i.e., no

oligodeoxynucleotide. As shown, the contractile response to NPY was markedly reduced after antisense treatment. Values represent the mean ± SEM; n represents 8-10, except for the mismatch where n represents 3; * represents $p < 0.05$; and ** represents $p < 0.01$ between sense and antisense (paired Wilcoxon signed rank test).

- - -

EXAMPLE I

Screening of a Human Fetal Brain cDNA Library

1. Human Fetal Brain cDNA Library:

The lambda ZAPII cDNA library (Stratagene) was made from mRNA of a human female fetal (17-18 week gestation) brain, using both oligo (dT) and random-sequence primers.

2. Transfer to Nylon Membranes:

After titering the fetal brain lambda ZAP cDNA library (10^9 pfu/ml), aliquots containing 50,000 phage particles were mixed with 0.2 ml of the host bacteria (XL1), which were then infected by incubating the mixture for 20 minutes at 37°C. In total, 200,000 clones were screened. Next, 6.5 ml top agarose (0.6%, at about 50°C) were added to the aliquots and poured onto 150 mm agar plates warmed to 37°C. The plates were incubated at 37°C for about 6-8 hours or until the plaques were confluent. The plates were cooled at 4°C for 2 hours before applying nylon filters. Biotrans nylon membrane (ICN) were placed onto the surface of the top agar, and markings were made with a syringe needle containing radioactive India ink for identification and orientation purposes. The filters were submerged in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 2 min and in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, ph 8.0) for 5 minutes, and then rinsed in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, ph 7.0). The filters were dried on Whatman 3MM paper and DNA was fixed to the filters by either using a UV Stratalinker 1800 (Stratagene) or baking at 80°C for 2 hours in a vacuum oven.

3. Probe Preparation:

A 500-bp PCR product, corresponding to part of the coding region (547-1047) of the rat orphan receptor was used to screen the human

fetal brain cDNA library. This probe was generated using the following
PCR conditions: 5 min at 95°C for 1 cycle, then 1 min at 93°C, 1 min at
45°C and 2 min at 72°C for 35 cycles, with the fetal brain cDNA library
as template, and a 23-mer forward primer

5   (TTCCAAAATGTATCACTTGCGGC, positions 547-569) and a 25-mer
reverse primer (TAGTCTCGTAGTCCGTCCGTCTCGAG, positions 1023-
1047). Both primers were synthesized on a Biosearch Cyclone DNA
Synthesizer. The PCR reaction contained 50 mM KCl, 10 mM Tris-HCl
(pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1.6 mM dNTPs (US

10   Biochemical), 50 pmol of forward and reverse primers, and 1 unit Taq
DNA Polymerase in a 100 μl reaction volume.
       4. Probe Labeling:
              The human fetal brain probe was labeled using a random primed
DNA labeling kit (Boehringer Mannheim Biochemical), following the

15   manufacturer's instructions. Approximately 25 ng of the human fetal
brain PCR product was heat denatured (10 minutes at 95°C), and the
following components were added: dATP, dGTP, dTTP mixture (all 0.5
mmol/l in Tris buffer); reaction mixture (10X buffer with random
hexamer primers); 50 μCi [alpha-^{32}P] dCTP, 3000 Ci/mmol; and 1 unit

20   Klenow enzyme. This mixture was incubated for 30 minutes at 37°C,
and heated at 65°C for 10 minutes to stop the reaction. The probe was
then purified by Sephadex G-50 Sin Columns to remove non-incorporated
deoxyribonucleotide triphosphates. The Pharmacia Oligolabelling Kit
was also used to label the human fetal brain probe.

25   5. Hybridization Conditions:
              The filters were prehybridized for 2 hours at 42°C in 25%
formamide, 1 M NaCl, 10% dextran sulfate, 5X Denhardt's solution and
1% SDS. The hybridization was carried out in the same solution with
the addition of the ^{32}P-labeled human fetal brain probe (300 μl volume,

30   200-300 cps/μl) at 42°C for 16 hours. The filters were then washed
twice for 5 minutes at room temperature in 2X SSC, 0.2% SDS and twice
at 42°C for 30 minutes in 2X SSC, 0.5% SDS. The nylon membranes were
exposed to XAR-5 (Kodak) film at -70°C for 24-72 hours.
       6. Secondary Screening:

7

The positive plaques were removed from the plates and placed in SM buffer ((0.1 M NaCl, 0.01 M MgSO$_4$, 50 mM Tris-HCl (pH 7.5), and 0.01% gelatin)). These plaques were diluted and titered with XL1 cells to yield about 10 plaques for the first set and 100 plaques for the

5   second set on 100 mm agar plates. As before, the plates were incubated at 37°C overnight and transferred to nylon membranes as previously described. The same prehybridization/hybridization conditions in the initial screening were also used for the secondary screening. Positive clones were chosen for the tertiary screening,

10   which was carried out essentially as described for the secondary screen.

       After isolating single positive plaques from the human fetal brain library, 8 were chosen for further restriction enzyme and sequence analysis. The next step is to sequence these cDNA clones to

15   determine whether any of them are homologous to the rat receptor, and if so, whether any of these candidates are full length clones, which is critical for functional expression studies of potential NPY/PYY receptor cDNAs.

20   EXAMPLE II

                      Sequencing cDNA Clones

1. In vivo Excision of pBluescript Plasmid from Lambda ZAP Vector:

       Phagemids were rescued from the lambda vector and transfected into XL1 Blue bacteria according to the Stratagene protocol. The single

25   positive plaques from the agar plates were removed and placed into a solution containing SM buffer and chloroform in Eppendorf tubes. The samples were incubated at room temperature for one hour, with occasional vortexing, after which 0.2 ml of the plaque samples were added to 0.2 ml XL1-Blue cells (OD$_{600}$ =1.0) and 1 µl of R408 helper

30   phage, and this was incubated at 37°C for 15 minutes. The 5 ml of 2X YT media (10 g NaCl, 10 g Yeast extract and 16 g Bacto-Tryptone/liter) were added to the samples and incubated for another 3 hrs at 37° C. Next, the samples were heated at 70° C for 20 minutes and centrifuged at 4000 x g for 5 min. The supernatant, containing the pBluescript

8

phagemid, was collected and 10 μl was removed, and combined with
200 μl XL1-Blue cells (OD$_{600}$ = 1.0), which was incubated at 37° C for
15 min. Subsequently, 20 and 50 μl of these transfected cells were
plated onto 100 mm LB/ampicillin plates and incubated overnight at 37°
5   C.

2. Plasmid DNA Purification:

The Bluescript vector was purified from colonies using Promega
Magic Miniprep system. The minipreps were performed according to the
manufacturer's protocol. Overnight cultures were pelleted by
10   centrifugation, and the pellets were resuspended in Cell Resuspension
Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μl/ml RNase A).
Cell lysis solution (0.2 M NaOH, 1 mM EDTA) was added to the
resuspended cells, and then the cells were neutralized in a solution of
2.55 M KOAc, pH 4.8. After spinning the samples in a microcentrifuge
15   (14,000 x g for 5 min), the supernatant was collected and the DNA
purification resin (Promega) was added before application to the mini-
column. The mini-colum,n containing the DNA-bound resin was rinsed
with Column Wash Solution (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5
mM EDTA and 50% ethanol); afterwards it was placed in a
20   microcentrifuge tube, which was spun quickly to dry the resin. The
plasmid DNA was eluted with pre-heated water (65-70°C) and respun,
and the purified DNA was collected. After restriction enzyme
characterization of the plasmid DNA, suitable clones were chosen for
sequence analysis.

25   2.1 Manual Sequencing Procedure:

Prior to the sequencing reaction, the double-stranded Bluescript
plasmid obtained from the miniprep procedure was alkali denatured
(incubation at 37° C for 30 min. in 0.2 M NaOH, 0.2 mM EDTA),
neutralized in 0.4 volume 5 M NH$_4$Ac and precipitated with 4 volume
30   100% ethanol at -70° C for 5 min., after which it was spun in a
microcentrifuge and the pellet was washed with 70% ethanol. The
sequencing was performed using The Sequenase Version 2.0 Sequencing
kit (US Biochemical) and the sequencing reactions were carried out
according to the manufacturer's instructions. Sterile water was added

to the pelleted DNA, resuspended, and the following components were
also added: 5X sequencing buffer (200 mM Tris-HCl, pH 7.5, 100 mM
MgCl$_2$, 250 mM NaCl), 3-4 pmol primer (T3, T7, SK, KS, M13 and M13 rev
were synthesized on an ABI 394 DNA/RNA Synthesizer). The primers

5 were annealed to the plasmid by heating at 65°C for 2 min. and then
cooled at room temperature. Each of the four termination mixtures
were pipetted into microtiter plate wells. The 5X labeling Mix (7.5 um
each of dGTP, dCTP, dTTP) was diluted with water, and the Sequenase
was diluted in the enzyme dilution buffer (10 mM Tris-HCl, pH 7.5, 5 mM

10 DTT and 0.5 mg/ml BSA). For the labeling reaction, 0.1 M DTT, diluted
labeling mix, 5 μCi [^{35}S]-dATP (Amersham, >1000 Ci/mmol) and diluted
sequenase were added to the annealing mixture and incubated at room
temperature for 5 min. For the termination reaction, the labeling
reaction was added to each of the termination mixtures, and incubated

15 for 5 min at 37° C. After this incubation, stop solution (95%
formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene
cyanol) was added to each reaction. The sequencing reactions were also
carried out with the Pharmacia T7 sequencing kit using their
sequencing protocol, which is similar to the procedure described above.

20 3. Automated Fluorescence Based Solid Phase Sequencing:
        Sequencing of the HY1-5 and HY1-7 clones were performed by
using the manual dideoxy chain termination reaction using T7 DNA-
polymerase and ^{35}S-ATP (described above) and by using a Taq-
polymerase based dideoxy chain termination reaction with dye-labeled

25 2',3'-dideoxynucleoside triphosphates, where the sequencing reaction
is separated on an automated DNA sequencing apparatus (Applied
Biosystems 373A Sequencing System) that automatically collects
sequence data and makes it possible to export sequences to a databank,
where further analysis of the sequence can take place. The procedure

30 for sequencing the human Y1 receptor by using Taq-polymerase based
dideoxy chain termination reactions with dye-labeled 2',3'-
didoxynucleoside triphosphates follows the protocol described below.
All PCR reactions were performed on thermal cyclers from Perkin-
Elmer.

3.1   Insert PCR of a DNA cloned in a plasmid and binding to a solid phase:

Two oligonucleotides (primers), JS1 (5'-GCGCGGATAACAATTTCACACA-3') and JS2 (5'-
5   GCAGCACTGACCCTTTTGGGACCG-3') were constructed. They correspond to the sequences juxtaposed to the linker of the PUC plasmid and its derivatives, making it possible to do PCR amplification of a DNA cloned in the plasmid's linker. A second set of the JS-primers, called JS1B and JS2B, were modified by coupling biotin to the primer's 5'-end. Biotin is
10   a protein that strongly binds a 66 kDa protein called Streptavidin. A PCR-reaction where one primer is biotinylated and the other is not generates a product that can be bound to a solid phase, in our case the Dynalbeads M-280 (ny Dynal, Norway) complexes between superparamagnetic polystyrene beads chemically bound to Streptavidin.
15   Once the biotinylated product is bound to streptavidin the product can be denatured and the non-bound DNA can be washed away, resulting in single stranded DNA bound to the magnetic beads. The standard PCR insert amplification protocol is:

Ten picomoles of each primer (either JS1-JS2b or JS1B-JS2,
20   depending on each strand that shall be sequenced), 10-100 picograms of plasmid-DNA in a PCR reaction that contains 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl$_2$, 0.8 mM dNTPs (Pharmacia), and 1 unit of Taq polymerase (Perkin-Elmer) in a   50 µl reaction volume.

Binding of the PCR generated product and separation of the two
25   strands were achieved following the protocol:

1) Add 20 µl (0.2 mg) of the Dynalbeads to a magnetic Eppendorf stand. This will precipitate the beads immediately. Wash the Dynalbeads two times with a 200 µl SAMAG-solution (10 mM Tris-HCl, pH 7.0, 1 M NaCl, 0.1% Triton X-100).
30   2) Transfer the tubes with the beads to a non-magnetic stand. Add 20 µl of the PCR mixture with the biotinylated product and incubate on a shaker for 30 minutes at room temperature.

3) Transfer the tubes to a magnetic stand. Wash the beads once with 200 µl SAMAG.

11

4) Transfer the tubes with the beads to a non-magnetic stand. Denature the DNA by adding 200 μl denaturation solution (0.1 M NaOH, 1 m NaCl). Incubate 15 minutes at room temperature.

5) Transfer the tubes to a magnetic stand. Wash once with 200 μl of the denaturing solution. Wash once with 100 μl of a 5 x PCR buffer (400 mM Tris-HCl pH 8.9, 100 mM Ammonium Sulfate, 25 mM $MgCl_2$) diluted 1:5.

6) Transfer the tubes with the beads to a non-magnetic stand. Dissolve each sample in 6 μl of ddH_2O. Aliqout the dissolved beads into four tubes marked A (1 μl), C (1 μl), G (2 μl), and T (2 μl). (See below "B. Aliquoting the reagents")

3.2   Fluorescent DNA Taq sequencing:

The dye primers, M13, M13rev, T3 and T7 and Taq-sequencing kit were purchased from ABI. Sequencing reactions were performed according to the manufacturer's protocol.

A. Diluting the enzyme

Mix 0.5 μl AmpliTaq DNA polymerase (8 units/μl) with a 1.0 μl 5 x PCR buffer (400 mM Tris-HCl pH 8.9, 100 mM Ammonium Sulfate, 25 mM $MgCl_2$) and 5.5 μl H_2O.

B. Aliquoting the reagents

Aliquot the reagents into four 0.5 ml microcentrifuge tubes according to the following protocol:

Reagent	A	C	G	T
d/ddNTP Mix	1 μl	1 μl	2 μl	2 μl
Dye primer (0.4 pmol/μl)	1 μl	1 μl	2 μl	2 μl
5 x PCR buffer	1 μl	1 μl	2 μl	2 μl
DNA template	1 μl	1 μl	2 μl	2 μl
Diluted Taq	1 μl	1 μl	2 μl	2 μl
Total Volume	5 μl	5 μl	10 μl 1	10 μl

Overlay each of the four reactions with about 20 μl mineral oil.

C. Cycling the reactions

Place the tubes in a thermal cycler preheated to 95°C.   For the present invention, a modified PCR cycle was used which included 1

12

minute at 94°C, 1 min at 55° C, and 1 min at 72° C for 25 cycles followed by a soak file at 4° C. These PCR conditions appeared to be as efficient as the conditions recommended by the manufacturer.

D. Concentrating the sample

5   In a separate tube mix 80 µl 95% ethanol with 1.5 µl 3 M sodium acetate (pH 5.3). Pipette the extension reaction from the bottom of each of the four tubes into the ethanol mixture. Mix thoroughly. Precipitate at room temperature for 10-15 minutes. Spin in a microcentrifuge for 30 minutes. Rinse the pellet with 70% ethanol and

10   spin for another 5 minutes. Dry the pellet in a vacuum centrifuge for 1-3 minutes.

E. Loading the sample

          Prior to loading, samples were resuspended in 6 µl of deionized formamide/50 mM EDTA (pH 8.0) in the proportions 5:1. Heat the sample

15   at 90° C for 2 min. and load immediately on a pre-electrophoresed gel.

3.3 The ABI 373A Apparatus

          The apparatus is based on a four-dye, one lane, scanned laser technology. Conventional 6% polyacrylamide gels are used ((57 g acrylamide, 3 g bis-acrylamide, 450 g urea and 100 ml 10 x TEB)/liter).

20   The settings on the machine to perform a 14 hour long run are based on the manufacturer's recommendations: 2500 V, 40 mAMP, 30 W, 40°C.

          The chromatograms obtained after a gel run on the ABI 373A (equivalent to the autoradiographs when reactive isotopes are used) were examined in detail by using Seqed™, the Macintosh compatible

25   software from ABI, that allows editing of the collected sequence. The sequences were then exported to a VAX computer that has access to the UWGCG (University of Wisconsin Genetics Computer Group) package and many sequence banks (e.g. GenBank, EMBL, Swiss-prot.). The sequences were transferred by using the shareware Xferit 1.5 by Falkenburg.

30   The alignment of all the sequences obtained from manual and automated sequencing was created as a project called HCY1 by using a program package consisting of e.g. Gelstart, Gelenter, and Geloverlap. Gelassemble All computer work was done by using a Macintosh LC connected to a Localtalk net, allowing communication with the VAX

13

computer via the Public software Telnet MacTCP communication
program (NSCA Software Development).

4. Gel Electrophoresis:

5   The sequencing reactions were incubated at 75° C for 2 min
before they were run on 6% acrylamide gels (57 g acrylamide, 3 g bis-
acrylamide, 480 g urea/liter in 1X TBE buffer). The gels were 0.4 mm
thick and 30 cm x 38 cm in size. After the samples were run, the gel
was dried (1 hr at 80° C) with a gel dryer, and exposed to XAR-5 film
for 18-72 hrs.

10   Enzyme digestion of rescued plasmids revealed several
overlapping sibling clones, of which suitable clones, i.e. the longest
(hY1-5) and those containing overlapping coding regions, were selected
for sequencing analysis. In addition, 4 specific synthetic primers (3
forward primers: CTCTTGCTTATGGA/GGCTGTGA,

15   TATGTAGGTATTGCTGTGATTTG, TATACCACTCTTCTC/TT/CTGGTGCTG and
one reverse primer, CTGGAAGTTTTTGTTCAGGAAT/CCCA were used for
manual sequencing of the hY1-5 clones and its deletion constructs (Eco
RV, Xho, Nsi-Sac and Nsi-Eco RV). The clone of interest (hY1-5) was
then further characterized by Northern blot hybridization in order to

20   estimate its mRNA size, and by Northern blots and RT-PCR to examine
its distribution. Southern hybridizations were also performed to
determine the number of copies of this gene present in the human
genome.

- - -

25   EXAMPLE III
                    Northern and Southern Hybridization

1. Preparation of mRNA:

    The mRNA from several neuroblastoma cell lines (SK-N-MC, IMR,
SH-SY-5Y, LAN1, LAN2, LAN5, 1523, 2674) were purified by standard

30   guanidinium isothiocyanate/oligo (dT)-cellulose methods. Briefly,
cultured cells were homogenized in guanidinium thiocyanate
homigenization buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH
7.5, 1% beta-mercaptoethanol, 0.5% sodium lauryl sarcosinate) and the
resulting lysate was centrifuged (5000 x g, 20 min). After collecting

14

the supernatant, 0.1 vol 3M sodium acetate (pH 5.2) and 0.5 vol cold
100% ethanol were added and incubated on ice for 2 hrs. The nucleic
acid was pelleted by centrifugation (as above), and the pellet was
resuspended in a second guanidinium thiocyanate buffer (4 M
5   guanidinium thiocyanate, 0.1 M sodium acetate, pH 7.0, 1 mM DTT, 20
mM EDTA, pH 8.0). The nucleic acid was precipitated in 0.5 vol cold
100% ethanol and incubated at -20° C for 2 hrs. The nucleic acid was
pelleted as before, and precipitated twice more. The final pellet was
resuspended in 20 mM EDTA (pH 8.0) and 1 volume chloroform: 1-butanol
10   (4:1). This was recentrifuged as before and extraction with
phenol/chloroform/isoamyl alcohol was repeated. To precipitate the
RNA, 3 volumes of 4 M sodium acetate (pH 7.0) was added to the last
aqueous phase, incubated at -20° C for 2 hrs before centrifugation,
after which two more rounds of ethanol precipitation were carried out
15   and the RNA was dissolved in water. The total RNA was heated at 65° C
for 5 min before addition of loading buffer (20 mM Tris-HCl, pH 7.6, 0.5
M NaCl, 1 mM EDTA, pH 8.0, 0.1% sodium lauryl sarcosinate), which was
then applied onto oligo (dT)-cellulose columns. The columns were
washed with the loading buffer and the poly (A) RNA was eluted with
20   the elution buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.05
SDS) and the collected poly(A) was precipitated in 0.1 volume 3 mM
sodium acetate (pH 5.2) and 2.5 volumes cold ethanol. After incubation
at -20°C, this RNA was pelleted by centrifugation (10,000 x g for 30
min) and was dissolved in water.
25   2. Transfer of mRNA to Nylon Membranes:

The mRNA (few µg) was run on formaldehyde-containing agarose
gels and transferred to nylon filters by capillary elution and RNA was
crosslinked to the nylon membranes using a UV Stratalinker 1800
(Stratagne).
30   3. Hybridization Conditions:

The probe, a 1.4 kb XhoI-EcoRI fragment of hY1-5, was labeled
with ^{32}P as described earlier in Example I. The prehybridization
conditions were carried out as described for cDNA screening, except
that 2X SSC was used instead of 1 M NaCl. The filters were washed

15

using the previously outlined conditions (for cDNA screening), with the exception that the final two washes were done at 65° C in 0.2 X SSC and 0.1% SDS. The filters were exposed to film as described earlier.

4. Southern Hybridization:

5   The human leucocyte genomic DNA was prepared (by standard procedure) and digested with restriction enzymes. This DNA was then run on a 1% agarose gel and the DNA was transferred to filters as described before. The probe, hybridization and washes were also as described for the Northern hybridization.

10   The Northern hybridization showed that the hY1-5 fragment hybridized to a single 3.5 kb transcript in SK-N-MC, which was known to express Y1 receptors, whereas the probe failed to identify Y1 transcripts in several other neuroblastoma cell lines. The Southern hybridization results suggests that the genome contains a single Y1

15 receptor gene. In order to confirm that the cDNA clones were the human Y1 receptor, specific primers synthesized for sequencing the clones, were used in RT-PCR.

- - -

EXAMPLE IV
Reverse Transcription-PCR

20 1. RNA Preparation:

      A. The mRNA was prepared from SK-N-MC cells using the Fast Track mRNA Isolation Kit (Invitrogen), following their instructions. In brief, cells were washed in PBS, pelleted by centrifugation,

25 resuspended and lysed in lysis buffer (kit), and subsequently homogenized in a Dounce homogenizer. The lysate was passed through a 21 gauge needle several times, incubated at 45° C for 2 hrs, and added to pre-equilibrated oligo (dT) cellulose and incubated for another hour at room temperature with shaking. The oligo (dT) cellulose-bound

30 mRNA was pelleted by centrifugation and resuspended in binding buffer (kit); this was repeated three times before the samples were loaded onto spin columns and quickly spun to remove excess buffer. Next, the oligo (dT) cellulose was resuspended in elution buffer and respun, after which the eluted mRNA was collected and precipitated in 0.15 volume 2

16

M sodium acetate and 2.5 volumes 100% ethanol. The RNA was pelleted
and resuspended in elution buffer, and stored at -70° C until used.

B. Cytoplasmic RNA was prepared from human cultured
circumflex coronary artery smooth muscle cells. As before, cells were
5 washed in PBS, centrifuged, and resuspended in lysis buffer (50 mM
Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 0.5% NP-40; also added
RNase inhibitor). After incubating on ice for 5 min, the lysate was
centrifuged, and the supernatant was collected. Proteinase K (100 µg
in 100 mM Tris, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS) was added
1 0 to the supernatant, and incubated for 15 min at 37° C. The RNA was
extracted with 2 sets of phenol/chloroform/isoamyl alcohol
extractions and precipitated with 1 volume isopropanol. The
cytoplasmic RNA was pelleted by centrifugation and redissolved in
water.

1 5 2.   Reverse Transcription Reaction:

The reverse transcription reaction was performed using the cDNA
Cycle kit (Invitrogen). Approximately 1 µg of SK-N-MC mRNA (or 5 µg
of circumflex coronary artery smooth muscle cell total RNA) was used
in each reaction, which consisted of 10 mM MeHgOH, 0.1 M beta-
2 0 mercaptoethanol, 0.2 µg of oligo dT primer, RNase inhibitor, 5X buffer,
1 mM dNTPs and 5 units reverse transcriptase. For the total RNA
sample, the primer was first incubated at 65° C for 2 min before the
addition of the other reagents. The samples were incubated at 42° C for
one hour, followed by another incubation at 95° C for 3 min, after which
2 5 another 5 units of reverse transcriptase was added and the cDNA
synthesis was repeated. The resulting cDNA was used directly for PCR
analysis.

3.   Polymerase Chain Reaction:

The four primers synthesized for the sequencing reaction
3 0 (Example II) were also used for the PCR reaction. The same PCR
reagents were used as described in the probe preparation section
(Example I; 50 pmol of forward and reverse primers), however, the
cycling conditions were slightly different: 5 min at 95° C for 1 cycle,
then 1 min at 93° C, 2 min at 55° C and 2 min at 72° C for 35 cycles.

17

The resulting PCR products were run on 1.5% agarose gels along with molecular weight markers to estimate their sizes.

- - -

EXAMPLE V

Functional Expression

The cDNA of the human NPY Y1 receptor was transfected into COS 1 cells. This was done in order to establish the functional identity of our clone. Following the transfection procedure described below, the cells were studied with respect to: (i) radioreceptor binding using ^{125}I-peptide YY (PYY); (ii) cyclic AMP accumulation by use of radioimmunoassay kit (Advanced Magnetics); and (iii) $^{45}Ca^2$ influx into the cells from the extracellular space. The two latter so-called second messenger responses were elicited by stimulation of the cells with NPY. In all three types of assay, cells not transfected with the Y1 receptor cDNA were used as controls to verify that these COS 1 cells do not normally possess Y1 receptors.

All steps of the transfection procedure were carried out under a sterile hood with the exception of purification and ethanol precipitation of the plasmid. Dulbecco's Modified Eagle Media (DMEM) contains 1% Penicillin-Streptomycin in all procedures unless otherwise specified.

1. COES 1 cells, passages 6 to 17, were maintained in T75 flask in DMEM supplemented with 10% fetal calf serum (GIBCO-BRL) at 37° C and 95% humidity under 5% CO_2 avoiding confluency until used.

2. The day before transfections were performed, cells were trypsinized and washed with 25 ml of DMEM containing 10% NuSerum (Collaborative Research; Catalogue #5000) to completely remove trypsin. After trituration, cells were subcultured to the density of 220,000 cells per 35 mm plate in 2 ml DMEM with 10% NuSerum. Allow approximately 20 hours for cells to attach to plates under the same culture-incubator condition described above.

3. On the day of transfection, the purified plasmid (cDNA of human NPY Y1 receptor in PCDM8 vector (Invitrogen) was precipitated with ethanol and dissolved in sterile 20 mM HEPES buffer (pH 7.4) containing 150 mM

18

NaCl to the final concentration of 0.1 mg/ml.   Mix 2 μg plasmid with 15
μg DEAE-dextran (stock solution 50 mg/ml) in a volume less than 50 μl
in a polystyrene tube.   The final concentration of DEAE-dextran should
be 500 μg/ml after mixed with media for transfections.   Leave the
5   mixture at room temperature until the media for the transfection is
prepared (10 minutes).   At this concentration of plasmid, normally no
precipitation was formed, however, if any precipitate was visible, the
volume of buffer was increased to 100 μl.

4.   Combine 1.5 ml DMEM containing 10% NuSerum with chloroquin
10   phosphate (stock solution 75 mM) to the final concentration of 75 μM,
then add the media to the plasmid DEAE-dextran mixture, mix and lay it
over cells.   Typically the transfection mixtures were prepared in a
batch when it was performed in a number of 35 mm plates.   For
example, for 50 plates of 35 mm diameter, 100 μg plasmid was mixed
15   with 750 μl of 50 mg/ml DEAE-dextran, to which 75 ml DMEM media
with 10% NuSerum and 75 μM chloroquin phosphate was added.   After
thorough mixing by pipetting up and down, 1.5 ml of the mixture was
added to each plate.

5.   After incubation at 37° C and 95% humidity under 5% CO_2 for 3.5
20   hours, not exceeding 4 hours, cells were shocked by incubating in 2 ml
10% DMSO (in Hank's balanced salt solution with Mg^{2+} and Ca^{2+}) for 1
minute.   Cells were then washed with 3 ml DMEM containing 10%
NuSerum and further incubated for 64-72 hours in 7 ml DMEM with 10%
fetal calf serum under the incubator condition described above.

25   6.   For the transfection using 145 mm plates, cells were subcultured to
2.8 x 10^6/plate in 15 mk DMEM with 10% NuSerum.   Immediately before
transfection, the media was replaced with 9 ml of fresh one containing
75 μM chloroquion phosphate.   Transfection mixture for each plate
contained 25 μg plasmid and 100 μl 50 mg DEAE-dextran in 1 ml DMEM
30   with 10% NuSerum and 75 μM chloroquin phosphate.   The mixture was
then added to the cells by dropwise over the media and the plate was
gently swirled to achieve the uniform mixing.

7.   The cells grown in 145 mm plates were washed three times,
harvested and suspended in 50 mM ice-cold Tris-HCl buffer (pH 7.4)

19

with 5 mM EDTA and 1 mM β-mercaptoethanol and then homogenized
using Polytron (Brinkman; setting 6) for 10 sec. The homogenate was
centrifuged at 1,000 x g for 10 min using a swinging bucket rotor. The
supernatant was then subjected to ultracentrifugation at 100,000 x g

5   for 30 min. The resulting pellet was resuspended by Polytron
homogenizer in fresh binding buffer (137 mM NaCl, 5.4 mM KCL, 0.44 mM
KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 20 mM HEPES, 0.3% bovine
serum albumin and 0.01 % bacitracin; pH 7.4) and membranes from 2 X
10^6 cells were used per assay tube in a final volume of 0.4 ml. Samples

10   were then incubated with ^{125}I-peptide YY ((^{125}I-PYY (New England
Nuclear); 2200 Ci/mmol; 22°C; 100 min)). In displacement-type
experiments, 0.1 nM radioligand was used. The incubations were
terminated by centrifugation (Eppendorf Microfuge) for 2 min, followed
by washing of the pellets' surface by 1 ml ice-cold buffer. Pellets were

15   counted in a Packard gamma-counter. Binding data were analyzed using
LIGAND™ and KINETIC™ (Biosoft).

8. Cells grown in 35 mm dishes were used for $^{45}Ca^{2+}$ influx studies or
cAmp studies:

         A. $^{45}Ca^{2+}$ influx:

20   Prior to influx studies, transfected (60 hours earlier) and
control transfected COS 1 cells were washed three times with 1.5 ml of
the above described binding buffer solution fortified with 10 mM
glucose. $^{45}Ca^{2+}$ influx (Amersham Corp; 3-5 µM; 6-10 µCi) was
studied over 2 minutes with or without agonist. Final volumes were 1

25   ml and experiments were performed at room temperature with
solutions kept at 37° C prior to use. The uptake was terminated by
rapid aspiration of the incubation mixture. Cells were then washed four
times with ice cold buffer in order to remove the residual extracellular
radioactivity and lysed with 1 ml of lysis buffer (8 M urea, 3 M acetic

30   acid, and 2% Nonidet P-40). The amount of $^{45}Ca^{2+}$ influx into the cells
was estimated by counting the disintegrations per minute (DPM) of
lysate aliquots in a liquid scintillation system after mixing with
Formula 989 (New England Nuclear).

         B. Cyclic AMP accumulation determination:

20

Sixty hours after transfection (35 mm wells) cells were equilibrated in the HEPES-based buffer described above for one hour. Phosphodiesterase inhibitor, methylisobutylxanthine (500 µM) was present throughout the cAMP experiments. Ten minutes after addition

5   of 100 nM NPY, the cells were challenged with 5 µM forskolin. Fifteen minutes later, the reaction was terminated by adding 1 ml of ethanol to the 1 ml of incubation mixture. The cells were harvested from each well into individual tubes, the wells were washed with another 1 ml of ethanol, and the washings were combined. The cells were then

10   sonicated and left on ice for 10 minutes. Precipitated proteins were separated by centrifugation, the precipitates were washed once with 1 ml of ethanol, and the supernatants were combined. The final ethanol extract was evaporated under vacuum and the residue dissolved in assay buffer supplied with the cyclic AMP radioimmunoassay kit. Assay for

15   cyclic AMP was carried out using the non-acetylated protocol, precisely as described by the kit manufacturer.

- - -

As described above, thirteen human fetal brain cDNA clones hybridizing under low stringency conditions to a human PCR fragment

20   corresponding to the rat "orphan" receptor, FC5, were isolated from 200,000 screened clones. Eight of these were rescued from the phage vector and were found to share several restriction sites, yet displaying distinct insert sizes. The independent clones hY1-5 and hY1-7 were used for DNA sequencing. Clone hY1-5 had an insert of 2.1 kb that

25   included the entire coding region of the putative homolog of FC5. We found that clone hY1-5 contained 200 bp of 5' untranslated sequence. Preceding the ATG initiation codon at position 197, all three reading frames were interrupted by termination codons. No polyadenylation signal was found at the 3' end of clone hY1-5.

30   The Nucleotide sequence of the human Y1-receptor cDNA is depicted below:

```
CCTTCTTTAA TGAAGCAGGA GCGAAAAAGA CAAATTCCAA AGAGGATTGT    50
TCAGTTCAAG GGAATGAAGA ATTCAGAATA ATTTTGGTAA ATGGATTCCA    100
ATATGGGGAA TAAGAATAAG CTGAACAGTT GACCTGCTTT GAAGAAACAT    150
```

21

```
        ACTGTCCATT TGTCTAAAAT AATCTATAAC AACCAAACCA ATCAAA        196
        ATG AAT TCA ACA TTA TTT TCC CAG GTT GAA AAT CAT TCA 235
        GTC CAC TCT AAT TTC TCA GAG AAG AAT GCC CAG CTT CTG 274
        GCT TTT GAA AAT GAT GAT TGT CAT CTG CCC TTG GCC ATG 313
    5   ATA TTT ACC TTA GCT CTT GCT TAT GGA GCT GTG ATC ATT 352
        CTT GTC TCT GGA AAC CTG GCC TTG ATC ATA ATC ATC TTG 391
        AAA CAA AAG GAG ATG AGA AAT GTT ACC AAC ATC CTG ATT 430
        GTG AAC CTT TCC TTC TCA GAC TTG CTT GTT GCC ATC ATG 469
        TGT CTC CCC TTT ACA TTT GTC TAC ACA TTA ATG GAC CAC 508
    10  TGG GTC TTT GGT GAG GCG ATG TGT AAG TTG AAT CCT TTT 547
        GTG CAA TGT GTT TCA ATC ACT GTG TCC ATT TTC TCT CTG 586
        GTT CTC ATT GCT GTG GAA CGA CAT CAG CTG ATA ATC AAC 625
        CCT CGA GGG TGG AGA CCA AAT AAT AGA CAT GCT TAT GTA 664
        GGT ATT GCT GTG ATT TGG GTC CTT GCT GTG GCT TCT TCT 703
    15  TTG CCT TTC CTG ATC TAC CAA GTA ATG ACT GAT GAG CCG 742
        TTC CAA AAT GTA ACA CTT GAT GCG TAC AAA GAC AAA TAC 781
        GTG TGC TTT GAT CAA TTT CCA TCG GAC TCT CAT AGG TTG 820
        TCT TAT ACC ACT CTC CTC TTG GTG CTG CAG TAT TTT GGT 859
        CCA CTT TGT TTT ATA TTT ATT TGC TAC TTC AAG ATA TAT 898
    20  ATA CGC CTA AAA AGG AGA AAC AAC ATG ATG GAC AAG ATG 937
        AGA GAC AAT AAG TAC AGG TCC AGT GAA ACC AAA AGA ATC 976
        AAT ATC ATG CTG CTC TCC ATT GTG GTA GCA TTT GCA GTC 1015
        TGC TGG CTC CCT CTT ACC ATC TTT AAC ACT GTG TTT GAT 1054
        TGG AAT CAT CAG ATC ATT GCT ACC TGC AAC CAC AAT CTG 1093
    25  TTA TTC CTG CTC TGC CAC CTC ACA GCA ATG ATA TCC ACT 1132
        TGT GTC AAC CCC ATA TTT TAT GGG TTC CTG AAC AAA AAC 1171
        TTC AGA GAC TTG CAG TTC TTC TTC AAC TTT TGT GAT 1210
        TTC CGG TCT CGG GAT GAT GAT TAT GAA ACA ATA GCC ATG 1249
        TCC ACG ATG CAC ACA GAT GTT TCC AAA ACT TCT TTG AAG 1288
    30  CAA GCA AGC CCA GTC GCA TTT AAA AAA ATC AAC AAC AAT 1327
        GAT GAT AAT GAA AAA ATC TGA AAC TAC TTA TAG CCT ATG 1366
        GTC CCG GAT GAC ATC TGT TTA AAA ACA AGC ACA ACC TGC 1405
        AAC ATA CTT TGA TTA CCT GTT CTC CCA AGG AAT GGG GTT 1444
        GAA ATC ATT TGA AAA TGA CTA AGA TTT TCT TGT CTT GCT 1483
```

22

TTT TAC AGT TTT GAC CAG ACA TCT TTG AAG TGC TTT TTG 1522
TGA ATT TAC CAG 1534

Within this sequence, the structural gene for the Y-1 receptor consists of the sequence between nucleotide 197 and 1534. The deduced amino acid sequence of the human Y1-receptor taken from this cDNA sequence is:

```
Met Asn Ser Thr Leu Phe Ser Gln Val Glu Asn His Ser Val His
                  5                 10                    15
Ser Asn Phe Ser Glu Lys Asn Ala Gln Leu Leu Ala Phe Glu Asn
                 20                 25                    30
Asp Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu
                 35                 40                    45
Ala Tyr Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala
                 50                 55                    60
Leu Ile Ile Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val Thr
                 65                 70                    75
Asn Ile Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala
                 80                 85                    90
Ile Met Cys Leu Pro Phe Thr Phe Val Tyr Thr Leu Met Asp His
                 95                100                   105
Trp Val Phe Gly Glu Ala Met Cys Lys Leu Asn Pro Phe Val Gln
                110                115                   120
Cys Val Ser Ile Thr Val Ser Ile Phe Ser Leu Val Leu Ile Ala
                125                130                   135
Val Glu Arg His Gln Leu Ile Ile Asn Pro Arg Gly Trp Arg Pro
                140                145                   150
Asn Asn Arg His Ala Tyr Val Gly Ile Ala Val Ile Trp Val Leu
                155                160                   165
Ala Val Ala Ser Ser Leu Pro Phe Leu Ile Tyr Gln Val Met Thr
                170                175                   180
Asp Glu Pro Phe Gln Asn Val Thr Leu Asp Ala Tyr Lys Asp Lys
                185                190                   195
Tyr Val Cys Phe Asp Gln Phe Pro Ser Asp Ser His Arg Leu Ser
                200                205                   210
Tyr Thr Thr Leu Leu Leu Val Leu Gln Tyr Phe Gly Pro Leu Cys
                215                220                   225
Phe Ile Phe Ile Cys Tyr Phe Lys Ile Tyr Ile Arg Leu Lys Arg
                230                235                   240
Arg Asn Asn Met Met Asp Lys Met Arg Asp Asn Lys Tyr Arg Ser
                245                250                   255
Ser Glu Thr Lys Arg Ile Asn Ile Met Leu Leu Ser Ile Val Val
                260                265                   270
```

23

```
        Ala Phe Ala Val Cys Trp Leu Pro Leu Thr Ile Phe Asn Thr Val
                        275                 280                 285
        Phe Asp Trp Asn His Gln Ile Ile Ala Thr Cys Asn His Asn Leu
                        290                 295                 300
   5    Leu Phe Leu Leu Cys His Leu Thr Ala Met Ile Ser Thr Cys Val
                        305                 310                 315
        Asn Pro Ile Phe Tyr Gly Phe Leu Asn Lys Asn Phe Gln Arg Asp
                        320                 325                 330
        Leu Gln Phe Phe Phe Asn Phe Cys Asp Phe Arg Ser Arg Asp Asp
  10                    335                 340                 345
        Asp Tyr Glu Thr Ile Ala Met Ser Thr Met His Thr Asp Val Ser
                        350                 355                 360
        Lys Thr Ser Leu Lys Gln Ala Ser Pro Val Ala Phe Lys Lys Ile
                        365                 370                 375
  15    Asn Asn Asn Asp Asp Asn Glu Lys Ile
                        380
```

The predicted Y1-receptor sequence shows 93% identity to that
deduced from the rat FC5 clone, which is proposed to correspond to a
rat Y1-receptor. Of the 24 amino acid replacements, seven occur in the
20 N-terminal extracellular part and nine occur in transmembrane region
(TM) 4 and the following extracellular loop. The human Y1-sequence has
two additional amino acids as compared to its rat counterpart, one in
the N-terminal extension and one near the C-terminus. It is notable
that the sequence DRY (Asp-Arg-Tyr), which follows TM3 in most
25 receptors belonging to the G-protein-coupled receptor superfamily,
reads ERH (Glu-Arg-His) in the Y1 sequences of both human and rat.
Most other positions which are highly conserved in the receptor
superfamily are also conserved in the predicted Y1-sequences.

The peptides according to the present invention can be
30 synthesized by any suitable method, such as by exclusively solid-phase
techniques, by partial solid-phase techniques, by fragment condensation
or by classical solution addition. Synthetic Y1-receptor according the
to the present invention may also be entirely or partially synthesized
by recently developed recombinant DNA techniques, which may likely be
35 used for large-scale production.

For example, the techniques of exclusively solid phase synthesis
are set forth in "Solid Phase Peptide Synthesis" by Stewart and Young,
Freeman & Company, San Francisco (1969), and exemplified in US Patent

24

4,105,603; fragment condensation methods of synthesis are
exemplified in US Patent 3,972,859; and other available synthesis
protocols are exemplified in US Patents 3,842,067 and 3,862,925.

5   Synthesis by use of recombinant DNA techniques, for purposes of
the present invention, should be understood to include the suitable
employment of a structural gene coding for all or an appropriate section
of the Y1-receptor to transform a microorganism, using an expression
vector including an appropriate promoter and operator together with the
structural gene, and causing the transformed microorganism to express

10   the peptide or such a synthetic peptide fragment. For example, either
the complete cDNA sequence for the Y1-receptor peptide depicted above
or the structural gene sequence from nucleotide 197 to nucleotide 1534
may be used in recombinant techniques. A non-human animal may also
be used to produce the peptide by gene-farming using such a structural

15   gene or cDNA in the microinjection of embryos.

Such recombinant techniques are well known in the field of
biotechnology, and can be easily used given the description presented
herein.

When the peptides are not prepared using recombinant DNA

20   technology, they are preferably prepared using solid phase synthesis,
such as that described by Merrifield [see J. Am. Chem. Soc. 85:2149
(1964), although other equivalent chemical syntheses known in the art
can also be used as previously described.

The presence of Y1-receptor mRNA in various human cultured

25   cells was investigated by (1) Northern hybridizations using human Y1-
probe (Fig. 1) and (2) by RT-PCR using specific human Y1-primers (data
not shown). Both methods showed the human neuroblastoma cell line,
SK-N-MC to express Y1-receptors (Fig. 1); this particular cell line has
been viewed as a model system for studies on Y1-receptors. The size of

30   the single hybridizing transcript in SK-N-MC is approx. 3.5 kb. By
Northern hybridization, we failed to identify Y1-transcripts in several
other neuroblastoma cell lines, i.e. IMR (Fig. 1), SH-SY-5Y, LAN1, LAN2,
LAN5, 1523 or 2674 (not shown). By using one reverse and three
forward primers in RT-PCR, we confirmed the presence of the Y1-

25

receptor in SK-N-MC, and, in addition, PCR products of the same sizes
(350, 520 and 850 bp) were also detected in human cultured circumflex
coronary artery smooth muscle cells. The latter observation is in
agreement with previous suggestions that the Y1-receptor is expressed
5   in vasculature. The same RT-PCR protocol, which again yielded 350,
520 and 850 bp products when the human fetal brain library was used
as template, failed to yield any detectable product in the neuroblastoma
cell line, SK-N-BE(2), which is thought to express Y2-receptors.
Southern hybridization to human genomic DNA followed by high-
10   stringency washes (Fig.1) suggest that the genome contains a single Y1-
receptor gene.

    The insert of hY1-5 was transferred to the mammalian
expression vector, pCDM8, and used to transfect COS1 cells. Such
transfected cells were used for studies on (1) radioligand, i.e. ^{125}I-
15   PYY, binding and (2) second messenger, i.e. Ca^{2+} and cAMP, analyses. As
a negative control for all these assays, identical COS1 cells
transfected with the rat type-1 angiotensin receptor in the same pCDM8
expression vector were used; in such control cells little or no specific
^{125}I-PYY binding was observed, and no second messenger responses to
20   NPY or PYY.

    Radioligand binding assays in membranes prepared from the hY1-
5 transfected cells indicate that the clone encodes a protein with the
pharmacological characteristics typical of a Y1-receptor type. The
dissociation constant (K_d) was 0.86 ± 0.09 nM (n = 4; mean ± SEM),
25   assuming a single-site fit and equal affinity of (porcine) ^{125}I-PYY and
unlabeled (porcine) PYY. This kDa is similar to that observed in SK-N-
MC and other cell types. The pharmacological profile of ligands
competing for ^{125}I-PYY binding to the expressed clone, illustrated in
Fig. 2, is also consistent with that of a Y1-receptor. The potency series
30   of PYY ≥ NPY ≥ [Leu^{31}, Pro^{34}]NPY >> NPY2-36 > C2-NPY > (human) PP >
NPY13-36 > NPY18-36 was determined (Fig.2); similar rank orders of
potency have been observed in various vascular smooth muscle cells
[see Br. J. Pharmacol. 105:45 (1992)] and SK-N-MC [see Life Sci. 50 PL7-
Pl12 (1992)]. Human NPY was equipotent with porcine NPY (not shown).

Two second messenger responses frequently associated with Y1-receptors are influx of Ca^{2+}, which is not necessarily associated with activation of phosphoinositidase C, and inhibition of cAMP accumulation. Thus, 100 nm of NPY and PYY was found to accelerate in

5   flux of $^{45}Ca^{2+}$, as studied over 2 min, by 135 ± 17% and 157 ± 23% of control, respectively (mean ± SEM; n = 6; two different experiments; p<0.001) in hY1-5 transfected COS1 cells; this is similar to the case for endogenous Y1-receptors in SK-N-MC. Control transfected cells did not respond to either NPY or PYY (100 nM). Another well-established

10   characteristic of Y1-receptors, e.g. in SK-N-MC, is the coupling to reduced cAMP accumulation. Likewise, stimulation of the *de novo* expressed Y1-receptor by 100 nM NPY inhibited forskolin (5 µM) elevated accumulation of cAMP in the COS1 cells by 47 ± 55% (mean ± SEM; n = 6; similar results obtained in two different experiments; p <

15   0.01). In the latter experiments, in which the phosphodiesterase inhibitor, methylisobutylxanthine (500 µM) was present throughout, 100 nM NPY also reduced basal cAMP concentrations from 240 ± 14 to 123 ± 4% (pmol of cAMP per 35 mm well; means ± SEM; n = 6; p < 0.001).

        The heterologously expressed Y1-receptor described herein is

20   thus similar to the endogenous Y1-receptor in brain, and neuroblastoma [5,7] and vascular smooth muscle cells with respect to binding and second messenger properties [see NY Acad. Sci. 611:7 (1990)]. Sequence analysis strongly indicated that the Y1-receptor belongs to the G-protein-coupled receptor superfamily. The human Y1-sequence is,

25   however, only distantly related to the two proposed NPY receptors that have appeared in the literature very recently [see Mol. Pharmacol. 40:869 (1991), and J. Biol. Chem. 267:9 (1992)]. The portion of the sequence spanning the TM regions of hY1-5 shows only 21% and 23% identity with proposed bovine and Drosophila NPY receptors,

30   respectively; the Y1-sequence appears more closely related to tachykinin receptors (29% sequence identity) [see Ann. NY Acad Sci. 632:53 (1991)], and it is similar to the human somatostatin type 1 receptor (23% identity) [see Proc. Natl. Acad. Sci. USA 89:251 (1992)] as to the bovine and Drosophila NPY receptors. Highly divergent sequences

27

within ligand-receptor families have also been reported for subtypes of amine receptors, however, no other peptide has previously been found to have receptor subtypes which display the degree of sequence divergence that exists between human Y1 (hY1-5) and bovine LCR1 (and Drosophila
5 PR4). For example, the three mammalian tachykinin receptors and the two human somatostatin receptors are 58-67% and 55% identical, respectively, over the regions spanning the TM segments.

In summary, the present invention has described the cloning and identification of the human Y1-type NPY/PYY receptor. This receptor is
10 thought to be instrumental for the ability of NPY/PYY to induce vasoconstriction as well as several behavioral effects.

As stated previously, Neuropeptide Y (NPY) is the most commonly found neurohormonal peptide in the human body. Consequently, the effects of NPY in the organism are many and varied. NPY, like many
15 other messenger molecules, acts by stimulating specific receptor molecules on the cell surface. Previous work has shown that such receptor molecules are heterogenous and that sub-types of receptors thus exist. The receptor sub-type cloned according to the present invention is termed "Y1-receptor", and is widely believed to mediate
20 some of the most important functions of NPY:

(1) Vascular smooth muscle contraction - NPY is released from nerves surrounding blood vessels and is one of the most potent known pressor agents, thus increasing blood pressure in man; elevated levels of NPY have been observed in hypertensive patients;

25 (2) Sedation/anxiolysis - NPY is as powerful as a benzodiazepine, e.g. Valium, in inducing anticonflict behaviors in animals; in psychiatric patients suffering from major depression, the brain levels of NPY are reduced, and anxiety symptoms in these patients are inversely related to NPY levels; and

30 (3) Food intake - NPY has frequently been argued to be the most powerful stimulator of food intake and obesity ever studied in mammals; dysregulation of NPY systems have been suggested to exist in patients with eating disorders, i.e. anorexia nervosa/bulimia.

28

For these and other reasons, the human Y1-receptor according to the present invention has a potential pharmaceutical target; at present, no therapeutically useful drugs are known to interact with the Y1-receptor. Its isolation and cloning according to the present invention

5   should greatly aid in screening efforts and rational drug design aiming to identify novel drugs that may either stimulate, inhibit, or block the Y1-receptor. Such drugs may thus perhaps be useful in the treatment of, e.g., hypertension, depression and/or anxiety, and eating disorders of various kinds as well as obesity. Such screening protocols are well

10 known utilizing other receptors, and these protocols may easily be modified by those skilled in the art to incorporate the use of the Y1-receptor according to the present invention.

- - -

EXAMPLE VI
15   Production and Testing of Oligonucleotides
The oligonucleotides necessary to study the inhibition of the contractile effect of neuropeptide Y on human blood vessels were synthesized on a Biosearch Cyclone DNA Synthesizer following the manufacturer's instructions. Three oligonucleotides were prepared: (1)

20 an antisense 18-base oligonucleotide (designated as hY1-AS) corresponding to the human Y1 receptor amino-terminus, (2) a corresponding sense oligonucleotide sequence (designated as hY1-S), and (3) a 3-base mismatched antisense oligonucleotide (designated as hY1-MM). The sequences of these three oligonucleotides were:

25   hY1-S   5' - CAACATTATT TTCCCAGG - 3'
            hY1-AS   5' - CCTGGGAAAA TAATGTTG - 3'
            hY1-MM   5' - CCTGAGATAA TAAGGTTG - 3'
Following deprotection with 30% ammonium hydroxide using conventional protocols, the oligonucleotides were lyophilized and

30 redissolved in water. These oligonucleotides were then run on a 15% acrylamide gel to verify their sizes.
Subcutaneous arteries and veins from patients operated upon for non-vascular diseases were dissected in the beginning of the operation from the abdominal region and cut into cylindrical segments 2-3 mm

29

long. These segments were incubated in Dulbecco's Modified Essential
Medium (Sigma) supplemented with streptomycin (10,000 mg/ml),
penicillin (10,000 U/ml) with or without the test oligonucleotides at 1
μM. Each incubation was conducted for 48 hours at 37° C in humidified
5   5% carbon dioxide and 95% air.

The cylindrical segments were then mounted on two metal
prongs, one of which was connected to a force displacement transducer
(model FT03C) attached to a Grass Polygraph for continuous recording
of the isometric tension, and the other to a displacement device. The
10   mounted specimens were immersed in temperature controlled (37° C)
tissue bathes containing a buffer of the following composition (mM):
NaCl 119, $NaHCO_3$ 15, KCl 4.6, MgCl 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5, and
glucose 11. The solution was continuously gassed with 5% carbon
dioxide in oxygen giving a pH of 7.4. A tension of 4 mN was applied to
15   the vessel segments and they were allowed to stabilize at this level of
tension for 1.5 hours. The contractile capacity of each vessel segment
was examined by exposure to a potassium-rich (60 mM) buffer solution.
After another 45 minutes rest period, the following known agonists
were added to the vessels in cumulative doses: neuropeptide Y (Auspep,
20   Australia), neuropeptide Y_{13-16} (Bissendorf Biochemicals),
pro^{34}neuropeptide, noradrenaline (Sigma).

- - -

In the human subcutaneous arteries and veins examined on day 1
without preincubation, NPY, PYY and Pro34NPY had similar contractile
25   effects while NPY_{13-36} had no contractile effect upon the vessels
tested, thus clearly indicating that the contractions seen were
mediated by a Y1-receptor.

Despite the 48 hour incubation, the vessels responded with
powerful contractions to 60 mM KCl (3.09 ± 0.27 mN), with no
30   difference between the groups receiving or not receiving the
oligonucleotides describe above. The contractile responses to
neuropeptide Y did not differ between the untreated group (that group
receiving no oligonucleotides), the sense oligonucleotide-treated group,

30

or the vessels incubated with mismatched oligonucleotides either in
arteries of veins as depicted in Figure 3 and the following table:

Table 1

Effect of Antisense Oligonucleotide Treatment On Potency and Maximum
Contraction in Human Subcutaneous Arteries and Veins

	Maximum Contraction	Potency
Human subcutaneous artery		
Control (no oligonucleotide)	80.8 ± 13.7%	7.49 ± 0.38
Antisense	20.2 ± 6.8%*	7.13 ± 0.15
Sense	79.4 ± 21.7%	7.30 ± 0.19
Mismatch	88.7 ± 39.0	7.24 ± 0.26
Human subcutaneous vein		
Control (no oligonucleotide)	74.3 ± 7.7%	7.61 ± 0.14
Antisense	33.4 ± 5.7%*	7.26 ± 0.24
Sense	57.7 ± 9.3%	7.51 ± 0.21
Mismatch	63.9 ± 28.8%	7.78 ± 0.05

- - -

The maximum contraction (% of potassium-induced contraction)
was significantly reduced by antisense oligoncleotide treatment as
shown in the above table. In this table, potency is expressed as -log
concentration of agonist inducing half maximum concentration, and no
significant differences were seen in the potency values between the
groups (artery and vein). All values represent the mean ± SEM for 8 to
10 vessel segments, except for the mismatch value which represents 3
segments. The asterisk (*) represents a $p < 0.01$ between the sense and
antisense data according to the paired Wilcoxon signed rank test.

As seen in Table 1, in both arteries and veins treated with Y1
receptor antisense oligonucleotide the contractile responses to NPY
were markedly attenuated. This inhibition did not appear to be
competitive in nature, since the potency values were not different
between the groups. The responses to noradrenaline (10^{-9} - 10^{-4} M) or
60 mM KCl did not differ between the groups.

As seen, after a 48 hour incubation period with the antisense
oligonucleotide (1 μM), the contractile responses to NPY were markedly

31

reduced. This is believed to be due to reduced numbers of NPY-Y1 receptors. This finding, in turn, indicates that the contractile effect of NPY on human resistance vessels that are likely to be active in the regulation of vascular tone and blood pressure, is mediated primarily by

5   the cloned Y1 receptor. The selectivity of the antisense oligodeoxynucleotide molecule seems to be very high since treatment with the mismatched analogue, hY1-MM, with 3 out of 18 nucleotides mismatched, was without effect on NPY-evoked vasoconstriction. Moreover, the antisense oligonucleotide did not affect responses of the

10   vessels to noradrenaline or high K$^+$ depolarization.

The antisense oligonucleotides described herein or deemed to be equivalents hereof, may be used in diagnostics, therapeutics and as research reagents and kits. For example, the use of the antisense oligonucleotide compounds may represent a suitable research tool for

15   vascular pharmacology by which the functional characteristics of a number of cloned receptors may be examined. For therapeutic use, the antisense oligonucleotides according to the present invention is to be administered to an animal, especially a human, in which it is medically desired to specifically attenuate NPY-evoked vasoconstriction.

20   Administration of the antisense oligonucleotides according to the present invention may be by any acceptable means, however, it is most preferred that the administration take place intravenously into a blood vessel, either artery or vein, so as to deliver the oligonucleotide directly to the site of NPY receptors. Use of recognized

25   pharmacologically acceptable carriers may also be preferred as carriers, diluents, buffers and other functional classes well within the purview of those skilled in the formulation arts. The exact dosages of antisense oligonucleotides provided to a mammal to attenuate the NPY-evoked response in the mammal's blood vessels may vary across a broad

30   range, however, such dosages should be limited to that range which is sufficient to bring about the desired degree of attenuation based upon the method of administration, the urgency by which such attenuation is desired, the weight of the mammal, and the amount of the oligonucleotide in the total bolus of medication administered. Such

32

variables are well within the purview of those skilled in the compounding and administration arts, and thus uniqueness for the use of antisense oligonucleotides to the human NPY receptor is not to be predicated upon any specific amount of oligonucleotide being
5 administered to the mammal in which vasoconstriction inhibition is desired.

A list of the nucleic acid and amino acid sequences which comprise the present invention follows:

SEQUENCE LISTING

10 (1) GENERAL INFORMATION:

(i) APPLICANT: Claes R. Wahlestedt

(iii) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
15   (A) LENGTH: 1534 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CCTTCTTTAA TGAAGCAGGA GCGAAAAAGA CAAATTCCAA AGAGGATTGT   50
TCAGTTCAAG GGAATGAAGA ATTCAGAATA ATTTTGGTAA ATGGATTCCA  100
ATATGGGGAA TAAGAATAAG CTGAACAGTT GACCTGCTTT GAAGAAACAT  150
ACTGTCCATT TGTCTAAAAT AATCTATAAC AACCAAACCA ATCAAA       196
ATG AAT TCA ACA TTA TTT TCC CAG GTT GAA AAT CAT TCA     235
GTC CAC TCT AAT TTC TCA GAG AAG AAT GCC CAG CTT CTG     274
GCT TTT GAA AAT GAT GAT TGT CAT CTG CCC TTG GCC ATG     313
ATA TTT ACC TTA GCT CTT GCT TAT GGA GCT GTG ATC ATT     352
CTT GTC TCT GGA AAC CTG GCC TTG ATC ATA ATC ATC TTG     391
AAA CAA AAG GAG ATG AGA AAT GTT ACC AAC ATC CTG ATT     430
GTG AAC CTT TCC TTC TCA GAC TTG CTT GTT GCC ATC ATG     469
TGT CTC CCC TTT ACA TTT GTC TAC ACA TTA ATG GAC CAC     508
TGG GTC TTT GGT GAG GCG ATG TGT AAG TTG AAT CCT TTT     547
GTG CAA TGT GTT TCA ATC ACT GTG TCC ATT TTC TCT CTG     586
GTT CTC ATT GCT GTG GAA CGA CAT CAG CTG ATA ATC AAC     625
```

33

CCT CGA GGG TGG AGA CCA AAT AAT AGA CAT GCT TAT GTA 664

GGT ATT GCT GTG ATT TGG GTC CTT GCT GTG GCT TCT TCT 703

TTG CCT TTC CTG ATC TAC CAA GTA ATG ACT GAT GAG CCG 742

TTC CAA AAT GTA ACA CTT GAT GCG TAC AAA GAC AAA TAC 781

5   GTG TGC TTT GAT CAA TTT CCA TCG GAC TCT CAT AGG TTG 820

TCT TAT ACC ACT CTC CTC TTG GTG CTG CAG TAT TTT GGT 859

CCA CTT TGT TTT ATA TTT ATT TGC TAC TTC AAG ATA TAT 898

ATA CGC CTA AAA AGG AGA AAC AAC ATG ATG GAC AAG ATG 937

AGA GAC AAT AAG TAC AGG TCC AGT GAA ACC AAA AGA ATC 976

1 0   AAT ATC ATG CTG CTC TCC ATT GTG GTA GCA TTT GCA GTC 1015

TGC TGG CTC CCT CTT ACC ATC TTT AAC ACT GTG TTT GAT 1054

TGG AAT CAT CAG ATC ATT GCT ACC TGC AAC CAC AAT CTG 1093

TTA TTC CTG CTC TGC CAC CTC ACA GCA ATG ATA TCC ACT 1132

TGT GTC AAC CCC ATA TTT TAT GGG TTC CTG AAC AAA AAC 1171

1 5   TTC CAG AGA GAC TTG CAG TTC TTC TTC AAC TTT TGT GAT 1210

TTC CGG TCT CGG GAT GAT GAT TAT GAA ACA ATA GCC ATG 1249

TCC ACG ATG CAC ACA GAT GTT TCC AAA ACT TCT TTG AAG 1288

CAA GCA AGC CCA GTC GCA TTT AAA AAA ATC AAC AAC AAT 1327

GAT GAT AAT GAA AAA ATC TGA AAC TAC TTA TAG CCT ATG 1366

2 0   GTC CCG GAT GAC ATC TGT TTA AAA ACA AGC ACA ACC TGC 1405

AAC ATA CTT TGA TTA CCT GTT CTC CCA AGG AAT GGG GTT 1444

GAA ATC ATT TGA AAA TGA CTA AGA TTT TCT TGT CTT GCT 1483

TTT TAC AGT TTT GAC CAG ACA TCT TTG AAG TGC TTT TTG 1522

TGA ATT TAC CAG 1534

2 5   (2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1338 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
3 0 .   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATG AAT TCA ACA TTA TTT TCC CAG GTT GAA AAT CAT TCA 39

GTC CAC TCT AAT TTC TCA GAG AAG AAT GCC CAG CTT CTG 78

3 5   GCT TTT GAA AAT GAT GAT TGT CAT CTG CCC TTG GCC ATG 117

34

```
ATA TTT ACC TTA GCT CTT GCT TAT GGA GCT GTG ATC ATT  156
CTT GTC TCT GGA AAC CTG GCC TTG ATC ATA ATC ATC TTG  195
AAA CAA AAG GAG ATG AGA AAT GTT ACC AAC ATC CTG ATT  234
GTG AAC CTT TCC TTC TCA GAC TTG CTT GTT GCC ATC ATG  273
TGT CTC CCC TTT ACA TTT GTC TAC ACA TTA ATG GAC CAC  312
TGG GTC TTT GGT GAG GCG ATG TGT AAG TTG AAT CCT TTT  351
GTG CAA TGT GTT TCA ATC ACT GTG TCC ATT TTC TCT CTG  390
GTT CTC ATT GCT GTG GAA CGA CAT CAG CTG ATA ATC AAC  429
CCT CGA GGG TGG AGA CCA AAT AAT AGA CAT GCT TAT GTA  468
GGT ATT GCT GTG ATT TGG GTC CTT GCT GTG GCT TCT TCT  507
TTG CCT TTC CTG ATC TAC CAA GTA ATG ACT GAT GAG CCG  546
TTC CAA AAT GTA ACA CTT GAT GCG TAC AAA GAC AAA TAC  585
GTG TGC TTT GAT CAA TTT CCA TCG GAC TCT CAT AGG TTG  624
TCT TAT ACC ACT CTC CTC TTG GTG CTG CAG TAT TTT GGT  663
CCA CTT TGT TTT ATA TTT ATT TGC TAC TTC AAG ATA TAT  702
ATA CGC CTA AAA AGG AGA AAC AAC ATG ATG GAC AAG ATG  741
AGA GAC AAT AAG TAC AGG TCC AGT GAA ACC AAA AGA ATC  780
AAT ATC ATG CTG CTC TCC ATT GTG GTA GCA TTT GCA GTC  819
TGC TGG CTC CCT CTT ACC ATC TTT AAC ACT GTG TTT GAT  858
TGG AAT CAT CAG ATC ATT GCT ACC TGC AAC CAC AAT CTG  897
TTA TTC CTG CTC TGC CAC CTC ACA GCA ATG ATA TCC ACT  936
TGT GTC AAC CCC ATA TTT TAT GGG TTC CTG AAC AAA AAC  975
TTC CAG AGA GAC TTG CAG TTC TTC TTC AAC TTT TGT GAT 1014
TTC CGG TCT CGG GAT GAT GAT TAT GAA ACA ATA GCC ATG 1053
TCC ACG ATG CAC ACA GAT GTT TCC AAA ACT TCT TTG AAG 1092
CAA GCA AGC CCA GTC GCA TTT AAA AAA ATC AAC AAC AAT 1131
GAT GAT AAT GAA AAA ATC TGA AAC TAC TTA TAG CCT ATG 1170
GTC CCG GAT GAC ATC TGT TTA AAA ACA AGC ACA ACC TGC 1209
AAC ATA CTT TGA TTA CCT GTT CTC CCA AGG AAT GGG GTT 1248
GAA ATC ATT TGA AAA TGA CTA AGA TTT TCT TGT CTT GCT 1287
TTT TAC AGT TTT GAC CAG ACA TCT TTG AAG TGC TTT TTG 1326
TGA ATT TAC CAG 1338
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:

```
                    (A) LENGTH:        384 amino acids
                    (B) TYPE:          amino acid
                    (C) STRANDEDNESS:      single
                    (D) TOPOLOGY:      linear
     5      (ii) MOLECULE TYPE:       peptide

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
        Met Asn Ser Thr Leu Phe Ser Gln Val Glu Asn His Ser Val His
                     5                   10                  15
        Ser Asn Phe Ser Glu Lys Asn Ala Gln Leu Leu Ala Phe Glu Asn
     10             20                  25                  30
        Asp Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu
                    35                  40                  45
        Ala Tyr Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala
                    50                  55                  60
     15 Leu Ile Ile Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val Thr
                    65                  70                  75
        Asn Ile Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala
                    80                  85                  90
        Ile Met Cys Leu Pro Phe Thr Phe Val Tyr Thr Leu Met Asp His
     20             95                  100                 105
        Trp Val Phe Gly Glu Ala Met Cys Lys Leu Asn Pro Phe Val Gln
                    110                 115                 120
        Cys Val Ser Ile Thr Val Ser Ile Phe Ser Leu Val Leu Ile Ala
                    125                 130                 135
     25 Val Glu Arg His Gln Leu Ile Ile Asn Pro Arg Gly Trp Arg Pro
                    140                 145                 150
        Asn Asn Arg His Ala Tyr Val Gly Ile Ala Val Ile Trp Val Leu
                    155                 160                 165
        Ala Val Ala Ser Ser Leu Pro Phe Leu Ile Tyr Gln Val Met Thr
     30             170                 175                 180
        Asp Glu Pro Phe Gln Asn Val Thr Leu Asp Ala Tyr Lys Asp Lys
                    185                 190                 195
        Tyr Val Cys Phe Asp Gln Phe Pro Ser Asp Ser His Arg Leu Ser
                    200                 205                 210
     35 Tyr Thr Thr Leu Leu Leu Val Leu Gln Tyr Phe Gly Pro Leu Cys
                    215                 220                 225
        Phe Ile Phe Ile Cys Tyr Phe Lys Ile Tyr Ile Arg Leu Lys Arg
                    230                 235                 240
        Arg Asn Asn Met Met Asp Lys Met Arg Asp Asn Lys Tyr Arg Ser
     40             245                 250                 255
        Ser Glu Thr Lys Arg Ile Asn Ile Met Leu Leu Ser Ile Val Val
                    260                 265                 270
```

```
      Ala Phe Ala Val Cys Trp Leu Pro Leu Thr Ile Phe Asn Thr Val
                      275             280              285
      Phe Asp Trp Asn His Gln Ile Ile Ala Thr Cys Asn His Asn Leu
                      290             295              300
   5  Leu Phe Leu Leu Cys His Leu Thr Ala Met Ile Ser Thr Cys Val
                      305             310              315
      Asn Pro Ile Phe Tyr Gly Phe Leu Asn Lys Asn Phe Gln Arg Asp
                      320             325              330
      Leu Gln Phe Phe Phe Asn Phe Cys Asp Phe Arg Ser Arg Asp Asp
  10                  335             340              345
      Asp Tyr Glu Thr Ile Ala Met Ser Thr Met His Thr Asp Val Ser
                      350             355              360
      Lys Thr Ser Leu Lys Gln Ala Ser Pro Val Ala Phe Lys Lys Ile
                      365             370              375
  15  Asn Asn Asn Asp Asp Asn Glu Lys Ile
                       380
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 base pairs

20   (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25 CAACATTATT TTCCCAGG 18

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 base pairs

        (B) TYPE: nucleic acid

30   (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

  CCTGGGAAAA TAATGTTG 18

35 (2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

37

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CCTGAGATAA TAAGGTTG 18

5   Thus while I have illustrated and described the preferred
embodiment of my invention, it is to be understood that this invention
is capable of variation and modification, and I therefore do not wish to
be limited to the precise terms set forth, but desire to avail myself of
such changes and alterations which may be made for adapting the
10   invention to various usages and conditions. Such variations and
modifications, for example, would include the substitution of
structurally similar nucleic and amino acid sequences which function to
yield substantially similar activities to those specifically described
above. Thus, changes in sequence by the substitution, deletion,
15   insertion or addition of nucleic acids (in the DNA sequences), or
substitution of completely different antisense sequences which do not
substantially alter the function of those sequences specifically
described above, are deemed to be within the scope of the present
invention. Accordingly, such changes and alterations are properly
20   intended to be within the full range of equivalents, and therefore within
the purview of the following claims.
        Having thus described my invention and the manner and a process
of making and using it in such full, clear, concise and exact terms so as
to enable any person skilled in the art to which it pertains, or with
25   which it is most nearly connected, to make and use the same;

38

I claim:

    1. An antisense ꟷ'igonucleotide sequence, corresponding to the amino-terminus of the human Y1 receptor, that is 5' - CCTGGGAAAA TAATGTTG - 3', said sequence being further characterized as having the specific pharmacologic action of attenuating neuropeptide Y-evoked vasoconstriction in human arteries and veins.

    2. A method for attenuating neuropeptide Y-evoked contractile response in a mammalien blood vessel which comprises contacting human Y1 receptors affecting the contractile response with an antisense oligonucleotide to the receptor in an amount sufficient to bring about an attenuation of the neuropeptide Y-evoked response.

    3. A method according to Claim 2 in which the antisense oligonucleotide is 5' - CCTGGGAAAA TAATGTTG - 3'.

    4. A cDNA for the genetic encoding of the human neuropeptide Y/peptide YY Y-1 receptor which is

```
CCTTCTTTAA TGAAGCAGGA GCGAAAAAGA CAAATTCCAA AGAGGATTGT    50
TCAGTTCAAG GGAATGAAGA ATTCAGAATA ATTTTGGTAA ATGGATTCCA   100
ATATGGGGAA TAAGAATAAG CTGAACAGTT GACCTGCTTT GAAGAAACAT   150
ACTGTCCATT TGTCTAAAAT AATCTATAAC AACCAAACCA ATCAAA       196
ATG AAT TCA ACA TTA TTT TCC CAG GTT GAA AAT CAT TCA       235
GTC CAC TCT AAT TTC TCA GAG AAG AAT GCC CAG CTT CTG       274
GCT TTT GAA AAT GAT GAT TGT CAT CTG CCC TTG GCC ATG       313
ATA TTT ACC TTA GCT CTT GCT TAT GGA GCT GTG ATC ATT       352
CTT GTC TCT GGA AAC CTG GCC TTG ATC ATA ATC ATC TTG       391
AAA CAA AAG GAG ATG AGA AAT GTT ACC AAC ATC CTG ATT       430
GTG AAC CTT TCC TTC TCA GAC TTG CTT GTT GCC ATC ATG       469
TGT CTC CCC TTT ACA TTT GTC TAC ACA TTA ATG GAC CAC       508
TGG GTC TTT GGT GAG GCG ATG TGT AAG TTG AAT CCT TTT       547
GTG CAA TGT GTT TCA ATC ACT GTG TCC ATT TTC TCT CTG       586
GTT CTC ATT GCT GTG GAA CGA CAT CAG CTG ATA ATC AAC       625
CCT CGA GGG TGG AGA CCA AAT AAT AGA CAT GCT TAT GTA       664
GGT ATT GCT GTG ATT TGG GTC CTT GCT GTG GCT TCT TCT       703
TTG CCT TTC CTG ATC TAC CAA GTA ATG ACT GAT GAG CCG       742
TTC CAA AAT GTA ACA CTT GAT GCG TAC AAA GAC AAA TAC       781
```

```
GTG TGC TTT GAT CAA TTT CCA TCG GAC TCT CAT AGG TTG  820
TCT TAT ACC ACT CTC CTC TTG GTG CTG CAG TAT TTT GGT  859
CCA CTT TGT TTT ATA TTT ATT TGC TAC TTC AAG ATA TAT  898
ATA CGC CTA AAA AGG AGA AAC AAC ATG ATG GAC AAG ATG  937
AGA GAC AAT AAG TAC AGG TCC AGT GAA ACC AAA AGA ATC  976
AAT ATC ATG CTG CTC TCC ATT GTG GTA GCA TTT GCA GTC  1015
TGC TGG CTC CCT CTT ACC ATC TTT AAC ACT GTG TTT GAT  1054
TGG AAT CAT CAG ATC ATT GCT ACC TGC AAC CAC AAT CTG  1093
TTA TTC CTG CTC TGC CAC CTC ACA GCA ATG ATA TCC ACT  1132
TGT GTC AAC CCC ATA TTT TAT GGG TTC CTG AAC AAA AAC  1171
TTC CAG AGA GAC TTG CAG TTC TTC TTC AAC TTT TGT GAT  1210
TTC CGG TCT CGG GAT GAT GAT TAT GAA ACA ATA GCC ATG  1249
TCC ACG ATG CAC ACA GAT GTT TCC AAA ACT TCT TTG AAG  1288
CAA GCA AGC CCA GTC GCA TTT AAA AAA ATC AAC AAC AAT  1327
GAT GAT AAT GAA AAA ATC TGA AAC TAC TTA TAG CCT ATG  1366
GTC CCG GAT GAC ATC TGT TTA AAA ACA AGC ACA ACC TGC  1405
AAC ATA CTT TGA TTA CCT GTT CTC CCA AGG AAT GGG GTT  1444
GAA ATC ATT TGA AAA TGA CTA AGA TTT TCT TGT CTT GCT  1483
TTT TAC AGT TTT GAC CAG ACA TCT TTG AAG TGC TTT TTG  1522
TGA ATT TAC CAG 1534.
```

5. A cDNA according to Claim 4 which is the structural gene for
human neuropeptide Y/peptide YY Y-1 receptor and which is:

```
ATG AAT TCA ACA TTA TTT TCC CAG GTT GAA AAT CAT TCA  39
GTC CAC TCT AAT TTC TCA GAG AAG AAT GCC CAG CTT CTG  78
GCT TTT GAA AAT GAT GAT TGT CAT CTG CCC TTG GCC ATG  117
ATA TTT ACC TTA GCT CTT GCT TAT GGA GCT GTG ATC ATT  156
CTT GTC TCT GGA AAC CTG GCC TTG ATC ATA ATC ATC TTG  195
AAA CAA AAG GAG ATG AGA AAT GTT ACC AAC ATC CTG ATT  234
GTG AAC CTT TCC TTC TCA GAC TTG CTT GTT GCC ATC ATG  273
TGT CTC CCC TTT ACA TTT GTC TAC ACA TTA ATG GAC CAC  312
TGG GTC TTT GGT GAG GCG ATG TGT AAG TTG AAT CCT TTT  351
GTG CAA TGT GTT TCA ATC ACT GTG TCC ATT TTC TCT CTG  390
GTT CTC ATT GCT GTG GAA CGA CAT CAG CTG ATA ATC AAC  429
CCT CGA GGG TGG AGA CCA AAT AAT AGA CAT GCT TAT GTA  468
```

40

GGT ATT GCT GTG ATT TGG GTC CTT GCT GTG GCT TCT TCT 507
TTG CCT TTC CTG ATC TAC CAA GTA ATG ACT GAT GAG CCG 546
TTC CAA AAT GTA ACA CTT GAT GCG TAC AAA GAC AAA TAC 585
GTG TGC TTT GAT CAA TTT CCA TCG GAC TCT CAT AGG TTG 624
TCT TAT ACC ACT CTC CTC TTG GTG CTG CAG TAT TTT GGT 663
CCA CTT TGT TTT ATA TTT ATT TGC TAC TTC AAG ATA TAT 702
ATA CGC CTA AAA AGG AGA AAC AAC ATG ATG GAC AAG ATG 741
AGA GAC AAT AAG TAC AGG TCC AGT GAA ACC AAA AGA ATC 780
AAT ATC ATG CTG CTC TCC ATT GTG GTA GCA TTT GCA GTC 819
TGC TGG CTC CCT CTT ACC ATC TTT AAC ACT GTG TTT GAT 858
TGG AAT CAT CAG ATC ATT GCT ACC TGC AAC CAC AAT CTG 897
TTA TTC CTG CTC TGC CAC CTC ACA GCA ATG ATA TCC ACT 936
TGT GTC AAC CCC ATA TTT TAT GGG TTC CTG AAC AAA AAC 975
TTC CAG AGA GAC TTG CAG TTC TTC TTC AAC TTT TGT GAT 1014
TTC CGG TCT CGG GAT GAT GAT TAT GAA ACA ATA GCC ATG 1053
TCC ACG ATG CAC ACA GAT GTT TCC AAA ACT TCT TTG AAG 1092
CAA GCA AGC CCA GTC GCA TTT AAA AAA ATC AAC AAC AAT 1131
GAT GAT AAT GAA AAA ATC TGA AAC TAC TTA TAG CCT ATG 1170
GTC CCG GAT GAC ATC TGT TTA AAA ACA AGC ACA ACC TGC 1209
AAC ATA CTT TGA TTA CCT GTT CTC CCA AGG AAT GGG GTT 1248
GAA ATC ATT TGA AAA TGA CTA AGA TTT TCT TGT CTT GCT 1287
TTT TAC AGT TTT GAC CAG ACA TCT TTG AAG TGC TTT TTG 1326
TGA ATT TAC CAG 1338

6. The isolated peptide

Met Asn Ser Thr Leu Phe Ser Gln Val Glu Asn His Ser Val His
           5   10   15
Ser Asn Phe Ser Glu Lys Asn Ala Gln Leu Leu Ala Phe Glu Asn
           20   25   30
Asp Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu
           35   40   45
Ala Tyr Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala
           50   55   60
Leu Ile Ile Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val Thr
           65   70   75
Asn Ile Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala
           80   85   90

41

```
Ile Met Cys Leu Pro Phe Thr Phe Val Tyr Thr Leu Met Asp His
              95                  100              105
Trp Val Phe Gly Glu Ala Met Cys Lys Leu Asn Pro Phe Val Gln
              110                 115              120
Cys Val Ser Ile Thr Val Ser Ile Phe Ser Leu Val Leu Ile Ala
              125                 130              135
Val Glu Arg His Gln Leu Ile Ile Asn Pro Arg Gly Trp Arg Pro
              140                 145              150
Asn Asn Arg His Ala Tyr Val Gly Ile Ala Val Ile Trp Val Leu
              155                 160              165
Ala Val Ala Ser Ser Leu Pro Phe Leu Ile Tyr Gln Val Met Thr
              170                 175              180
Asp Glu Pro Phe Gln Asn Val Thr Leu Asp Ala Tyr Lys Asp Lys
              185                 190              195
Tyr Val Cys Phe Asp Gln Phe Pro Ser Asp Ser His Arg Leu Ser
              200                 205              210
Tyr Thr Thr Leu Leu Leu Val Leu Gln Tyr Phe Gly Pro Leu Cys
              215                 220              225
Phe Ile Phe Ile Cys Tyr Phe Lys Ile Tyr Ile Arg Leu Lys Arg
              230                 235              240
Arg Asn Asn Met Met Asp Lys Met Arg Asp Asn Lys Tyr Arg Ser
              245                 250              255
Ser Glu Thr Lys Arg Ile Asn Ile Met Leu Leu Ser Ile Val Val
              260                 265              270
Ala Phe Ala Val Cys Trp Leu Pro Leu Thr Ile Phe Asn Thr Val
              275                 280              285
Phe Asp Trp Asn His Gln Ile Ile Ala Thr Cys Asn His Asn Leu
              290                 295              300
Leu Phe Leu Leu Cys His Leu Thr Ala Met Ile Ser Thr Cys Val
              305                 310              315
Asn Pro Ile Phe Tyr Gly Phe Leu Asn Lys Asn Phe Gln Arg Asp
              320                 325              330
Leu Gln Phe Phe Phe Asn Phe Cys Asp Phe Arg Ser Arg Asp Asp
              335                 340              345
Asp Tyr Glu Thr Ile Ala Met Ser Thr Met His Thr Asp Val Ser
              350                 355              360
Lys Thr Ser Leu Lys Gln Ala Ser Pro Val Ala Phe Lys Lys Ile
              365                 370              375
Asn Asn Asn Asp Asp Asn Glu Lys Ile
      380
```

7. A method for screening compounds for the treatment of a condition brought about by other than normal clinical amounts of neuropeptide Y in a patient which comprises bringing said compound in

42

contact with an isolated human Y1-receptor peptide, or a fragment
thereof having Neuropeptide Y activity, having the amino acid sequence:

```
Met Asn Ser Thr Leu Phe Ser Gln Val Glu Asn His Ser Val His
              5                   10                  15
Ser Asn Phe Ser Glu Lys Asn Ala Gln Leu Leu Ala Phe Glu Asn
              20                . 25                  30
Asp Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu
              35                  40                  45
Ala Tyr Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala
              50                  55                  60
Leu Ile Ile Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val Thr
              65                  70                  75
Asn Ile Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala
              80                  85                  90
Ile Met Cys Leu Pro Phe Thr Phe Val Tyr Thr Leu Met Asp His
              95                  100                 105
Trp Val Phe Gly Glu Ala Met Cys Lys Leu Asn Pro Phe Val Gln
              110                 115                 120
Cys Val Ser Ile Thr Val Ser Ile Phe Ser Leu Val Leu Ile Ala
              125                 130                 135
Val Glu Arg His Gln Leu Ile Ile Asn Pro Arg Gly Trp Arg Pro
              140                 145                 150
Asn Asn Arg His Ala Tyr Val Gly Ile Ala Val Ile Trp Val Leu
              155                 160                 165
Ala Val Ala Ser Ser Leu Pro Phe Leu Ile Tyr Gln Val Met Thr
              170                 175                 180
Asp Glu Pro Phe Gln Asn Val Thr Leu Asp Ala Tyr Lys Asp Lys
              185                 190                 195
Tyr Val Cys Phe Asp Gln Phe Pro Ser Asp Ser His Arg Leu Ser
              200                 205                 210
Tyr Thr Thr Leu Leu Leu Val Leu Gln Tyr Phe Gly Pro Leu Cys
              215                 220                 225
Phe Ile Phe Ile Cys Tyr Phe Lys Ile Tyr Ile Arg Leu Lys Arg
              230                 235                 240
Arg Asn Asn Met Met Asp Lys Met Arg Asp Asn Lys Tyr Arg Ser
              245                 250                 255
Ser Glu Thr Lys Arg Ile Asn Ile Met Leu Leu Ser Ile Val Val
              260                 265                 270
Ala Phe Ala Val Cys Trp Leu Pro Leu Thr Ile Phe Asn Thr Val
              275                 280                 285
Phe Asp Trp Asn His Gln Ile Ile Ala Thr Cys Asn His Asn Leu
              290                 295                 300
```

43

```
Leu Phe Leu Leu Cys His Leu Thr Ala Met Ile Ser Thr Cys Val
            305                 310                 315
Asn Pro Ile Phe Tyr Gly Phe Leu Asn Lys Asn Phe Gln Arg Asp
            320                 325                 330
Leu Gln Phe Phe Phe Asn Phe Cys Asp Phe Arg Ser Arg Asp Asp
            335                 340                 345
Asp Tyr Glu Thr Ile Ala Met Ser Thr Met His Thr Asp Val Ser
            350                 355                 360
Lys Thr Ser Leu Lys Gln Ala Ser Pro Val Ala Phe Lys Lys Ile
            365                 370                 375
Asn Asn Asn Asp Asp Asn Glu Lys Ile
          380
```

and determining whether said compound stimulates, inhibits or blocks
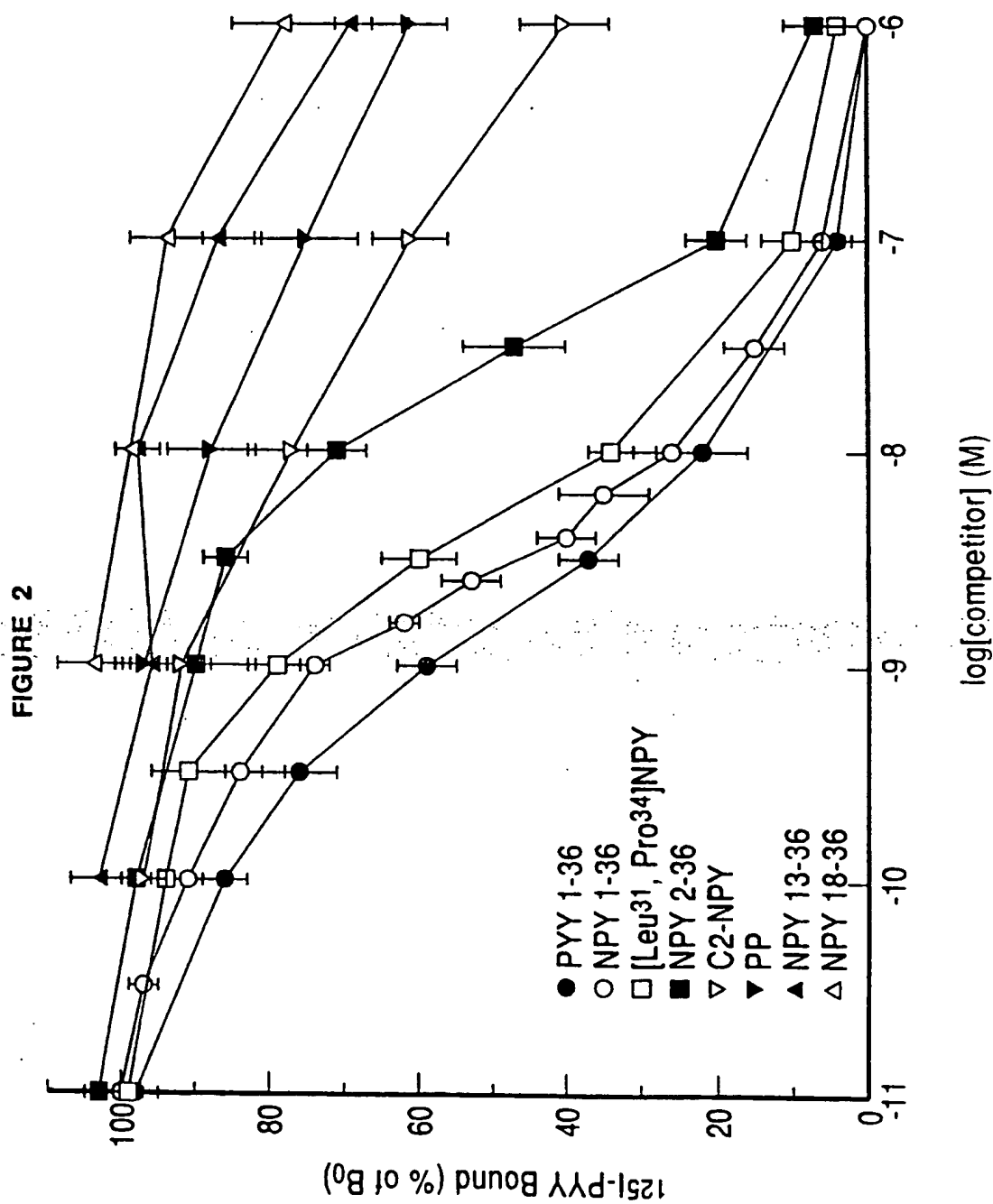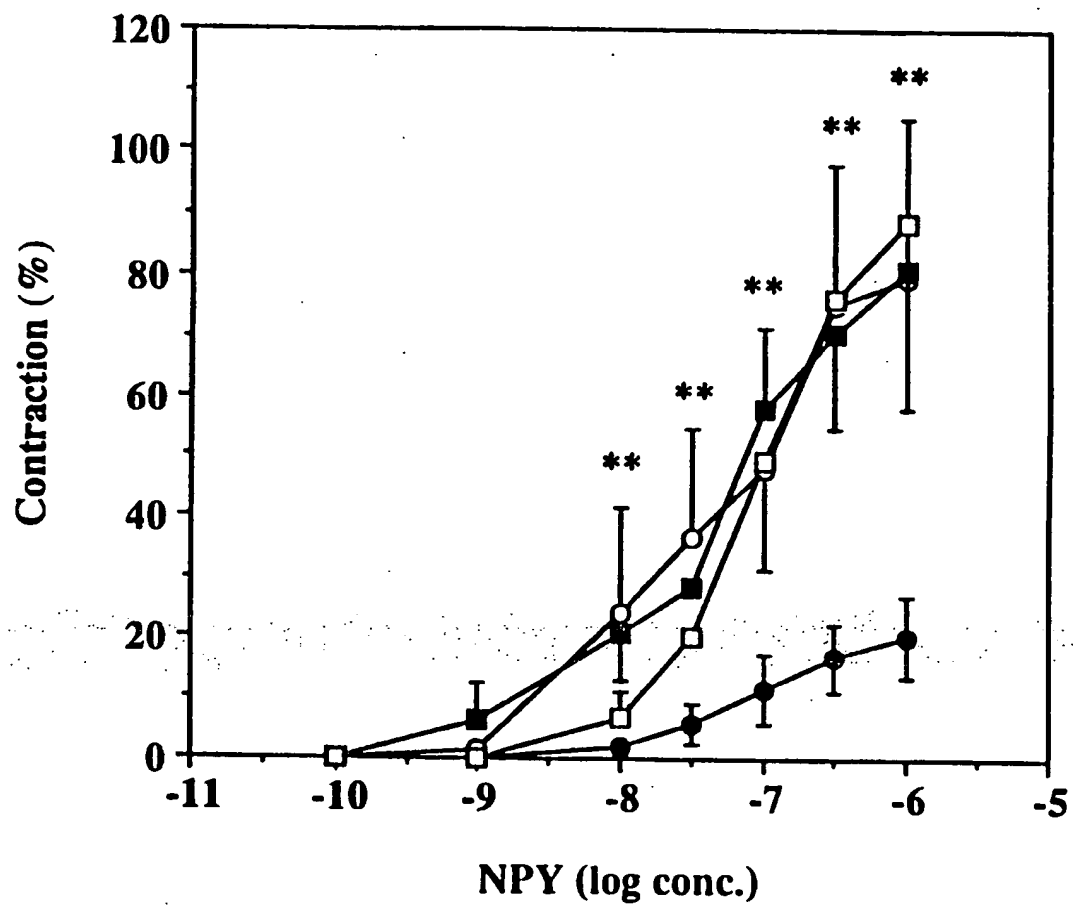the human Y1-receptor following conventional screening protocols.
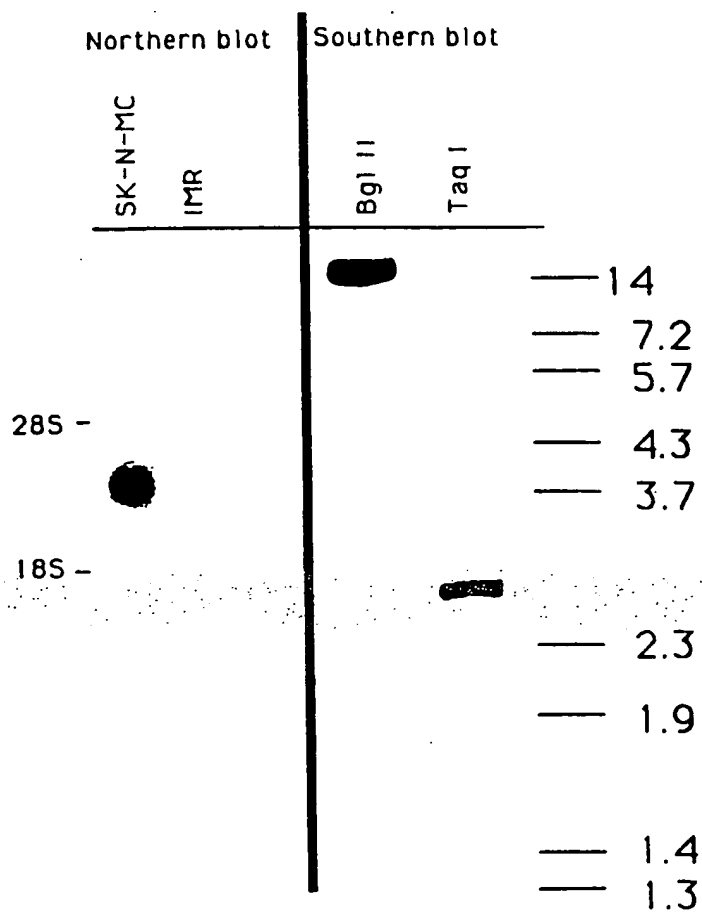
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4
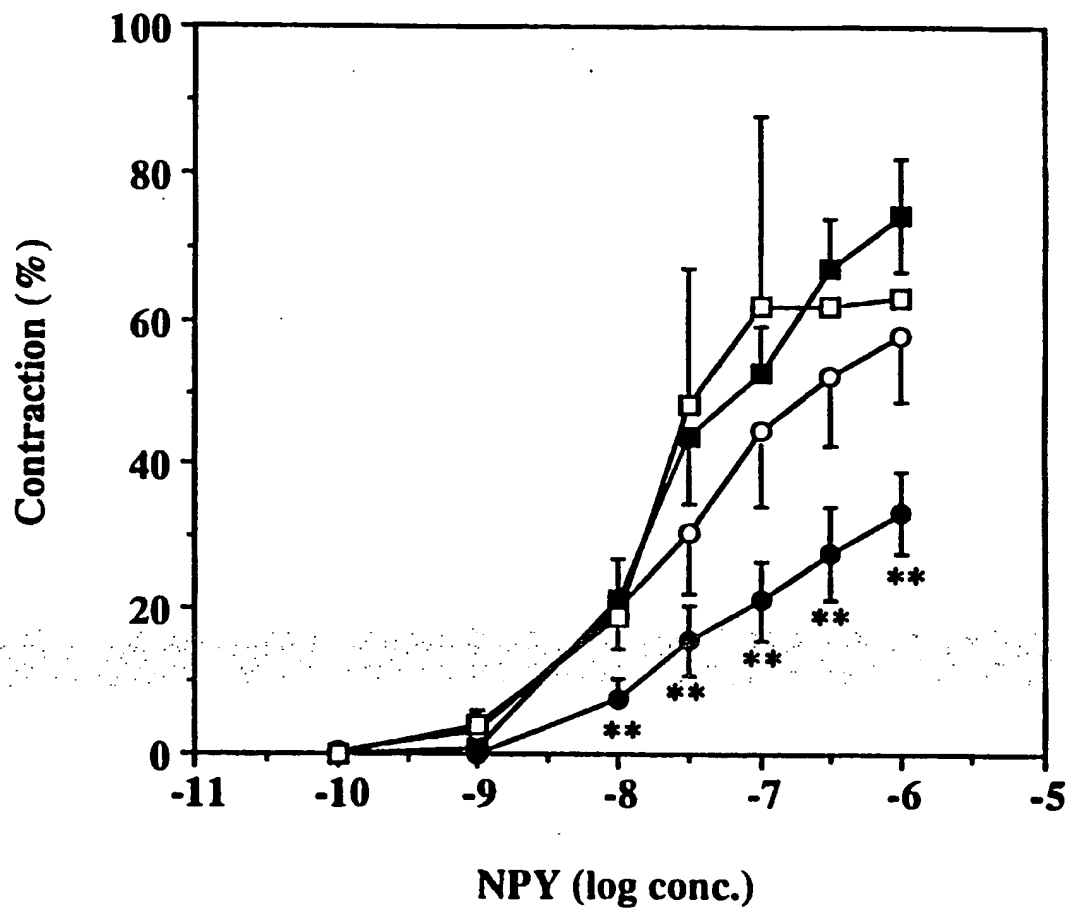
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5)   :C07H 21/04; C12N 1/21, 5/00; C12P 21/00
US CL   :536/23.1; 530/350; 514/12/ 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. :   536/23.1; 530/350; 514/12; 435/6
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Medline, Dialog
search terms: neuropeptide Y, peptide YY, receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Volume 267, No. 1, issued 05 January 1992, X. Li et al, "Cloning, Functional Expression, and Developmental Regulation of a Neuropeptide Y Receptor from Drosophila Melanogaster", pages 9-12, see abstract.	1-8
Y	European Journal of Pharmacology, Volume 204, issued 1991, H. N. Doods et al, "Different Neuropeptide Y Receptor Subtypes in Rat and Rabbit Vas Deferens", pages 101-103, see abstract.	1-8

☒ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

*	Special categories of cited documents:	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 AUGUST 1993	SEP 13 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SALLY P. TENG
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacolog, Volume 40, issued 1991, J. Rimland et al, "Sequence and Expression of a Neuropeptide Y Receptor cDNA", pages 869-875, see abstract.	1-8
Y	Annals of the New York Academy of Sciences, Volume 611, issued 01 December 1990, C. Wahlestedt et al, "Central and Peripheral Significance of Neuropeptide Y and its Related Peptides", pages 7-26, see pages 11-18.	1-8